AD_____

Award Number: DAMD17-98-C-8003

TITLE: Preparation of a Burkholderia Mallei Vaccine

PRINCIPAL INVESTIGATOR: Donald E. Woods, Ph.D.

CONTRACTING ORGANIZATION: Canadian Commercial Corporation
Ottawa, Ontario, Canada K1A OS6

REPORT DATE: January 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2001	Final (15 Dec 97 - 14 Dec 00)

4. TITLE AND SUBTITLE
Preparation of a Burkholderia Mallei Vaccine

5. FUNDING NUMBERS
DAMD17-98-C-8003

6. AUTHOR(S)
Donald E. Woods, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Canadian Commercial Corporation
Ottawa, Ontario, K1A OS6 Canada

E-MAIL:
woods@ucalgary.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Glanders, caused by *Burkholderia mallei*, is a significant disease for humans due to the serious nature of the infection. It is recognized that *B. mallei* is an organism with tremendous infectivity that poses a significant hazard to humans exposed to aerosols containing this organism. Our knowledge of the pathogenesis of disease due to *B. mallei* is lacking. At present, no effective vaccines are available against this organism, and information on the treatment of this organism with antibiotic therapy is also not available. The basic studies that we are performing on the pathogenesis of disease due to *B. mallei* are acutely needed, and the information gained from these studies will provide a knowledge base that is required to rationally design new modes of therapy directed against this organism. The long-term objective of our research is to define at a molecular level the pathogenesis of disease due to *B. mallei* and to Develop immunoprotective vaccines against these organisms.

14. SUBJECT TERMS
Burkholderia Mallei Vaccine, Biodefense

15. NUMBER OF PAGES
55

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

INTRODUCTION

Glanders, caused by *Burkholderia mallei*, is a significant disease for humans due to the serious nature of the infection. It is recognized that *B. mallei* is an organism with tremendous infectivity that poses a significant hazard to humans exposed to aerosols containing this organism. Our knowledge of the pathogenesis of disease due to *B. mallei* is lacking. At present, no effective vaccines are available against this organism, and information on the treatment of this organism with antibiotic therapy is also not available.

The basic studies that we are performing on the pathogenesis of disease due to *B. mallei* are acutely needed, and the information gained from these studies will provide a knowledge base that is required to rationally design new modes of therapy directed against this organism. The long-term objective of our research is to define at a molecular level the pathogenesis of disease due to *B. mallei* and to develop immunoprotective vaccines against these organisms for use in humans.

Since glanders is of military significance as a biological warfare agent, the development of an effective vaccine and treatments are of particular concern. Our understanding of the disease caused by *B. mallei* is minimal, and we must move forward with these studies in order to develop new and effective vaccines and/or therapies against this organism. There is considerable dual use potential, since this disease is important in various areas of the world. Development of vaccines and treatments can, therefore, provide important items to assist the World Health Organization and to assist signatories of the Biological and Toxin Weapons Convention under Article X of the Convention.

BODY

Detection of Bacterial Virulence Genes by Subtractive Hybridization

We have described a method combining PCR-based subtractive hybridization, insertional mutagenesis and an animal infection model for efficiently detecting such virulence genes, and we have applied the method to the pathogens *Burkholderia pseudomallei* and *B. mallei*. *B. pseudomallei*, the etiologic agent of melioidosis, is responsible for a broad spectrum of illnesses in humans and animals particularly in Southeast Asia and northern Australia, where it is endemic. *Burkholderia thailandensis* is an environmental organism closely related to *B. pseudomallei*, but is non-pathogenic. Subtractive hybridization was carried out between these two species in order to identify genes encoding for virulence determinants in *B. pseudomallei*. Screening of the library revealed A-T rich DNA sequences unique to *B. pseudomallei*, suggesting that they may have been acquired by horizontal transfer. One of the subtraction clones, pDD1015, encoded a protein with homology to a glycosyltransferase from *Pseudomonas aeruginosa*. This gene was insertionally inactivated in wild type *B. pseudomallei* to create SR1015. It was determined by ELISA and immunoelectron microscopy that the inactivated gene was involved in the production of a major surface polysaccharide. SR1015 was found to be severely attenuated for virulence in the Syrian hamster model of infection. The LD_{50} for wild type *B. pseudomallei* is <10. The LD_{50} for SR1015 was determined to be 3.5×10^5, similar to that of *B. thailandensis* (6.8×10^5). DNA sequencing of the region flanking the glycosyltransferase gene revealed open reading frames similar in sequence to capsular polysaccharide genes in *Haemophilus influenzae*, *E. coli*, and *Neisseria meningitidis*. In addition, *Burkholderia mallei* and *Burkholderia cepacia* showed reactivity in Southern blot analysis using the glycosyltransferase fragment as a probe, and a capsular structure was identified on the surface of *B. cepacia* via immunoelectron microscopy. The combination of PCR-based subtractive hybridization, insertional inactivation and animal virulence studies should facilitate the identification of relevant virulence determinants from a number of bacterial species.

A manuscript describing the studies detailed above is attached as Appendix 1.

TnphoA Mutagenesis of *Burkholderia mallei*

The objective of this work was to develop a system that facilitates the isolation of defined mutations in virulence genes of *B. mallei*. One such system is the *TnphoA* fusion vector. The *pho*A gene fusion approach takes advantage of the fact that for the normally periplasmic protein bacterial alkaline phosphatase (PhoA) to be active, it must be localised extracytoplasmically. The *TnphoA* system is composed of a truncated *phoA* gene (lacking the signal sequence) at one end of Tn5 forming a transposon, which can randomly generate gene fusions to *phoA* upon insertion into the recipient chromosome. The hybrid proteins expressed by such gene fusions exhibit PhoA activity only if the target gene encodes a membrane, periplasmic, outer membrane, or extracellular protein. Because exported proteins represent the most frequent classes of

proteins involved in pathogenesis; the use of *TnphoA* provides a strong enrichment for insertion into virulence genes. Selection of mutants carrying active *phoA* gene fusions is a simple procedure; colonies expressing alkaline phosphatase appear blue on agar containing the indicator 5-bromo-4-chloro-3-indolyl phosphate (XP).

Isolation and cloning of the *B. mallei* acid phosphatase gene. In order for the *TnphoA* procedure to be effective, the recipient organism must be acid phosphatase (AP) negative. Primers constructed to the AP gene of *B. pseudomallei* strain 1026b were used to amplify the AP gene from *B. mallei* GB8. The resulting PCR product was cloned into the TOPO cloning vector and sequenced; the DNA sequence was at least 94 % identical to the AP gene of 1026b.

Construction of a *B. mallei* acid phosphatase mutant. The pKAS46 allelic exchange vector (Km^R and RpsL) has been used successfully in the *B. pseudomallei* strain 1026b. We have previously used this vector to construct a plasmid (p463EZ) which contained an insertion inactivation in the 1026b AP gene utilising a zeocin cassette. Because of the high DNA homology (>94 %) between GB8 and 1026b AP genes an attempt was made to use (p463EZ) to inactivate the GB8 AP gene. One requirement of the pKAS46 vector is that recipient organisms are streptomycin (Sm) resistant. Unfortunately, spontaneous Sm^R *B. mallei* mutants were repeatedly also Sm dependent, and therefore would not grow in the absence of antibiotic. To overcome this problem, transconjugates from a GB8 and SM10λpir (p463EZ) mutagenesis were selected on XP, zeocin plates. 64/1500 transconjugates had lost AP activity, and 1/64 was Km sensitive, indicating a double crossover event had occurred and vector had been lost. The GB8 AP mutant (Ze^R, Km^S) mutant was designated G8P. *B. mallei* is resistant to Polymixin B at 15ug/ml; this concentration was routinely used to kill the *E. coli* donor strain following mutagenesis experiments. Unfortunately, due to the very low number of transconjugates obtained from the *TnphoA* mutagenesis, a high background of Polymixin B resistant (15ug/ml) *E. coli* occurred. To counteract this problem, a spontaneous Nalidixic acid resistant (75ug/ml) G8P was selected, designated G8PN.

***TnphoA* mutagenesis utilising pRT733(*TnphoA*).** Upon transfer of the suicide vector pRT733 carrying the *TnphoA* into G8PN, approximately 2% of transconjugants expressed PhoA activity (PhoA+), unfortunately, initial experiments with this system yielded very few transconjugates, 10-20 per mutagenesis. Mutagenesis conditions were optimized and the final procedure enabled ten mutagenesis' to be performed on one agar plate without the possibility of cross contamination; one selection plate was used per mutagenesis resulting in 100 to 200 transconjugates per mate. To prevent duplication of clones, generally one PhoA+ cfu was picked per mating. Transconjugates were selected on TSAG-DC agar (iron chelated media) and TSAG supplemented with 10%horse serum containing Nalidixic acid (75ug/ml), zeocin (5 ug/ml), Kanamycin (5 ug/ml) and XP (80 ug/ml).

Limitation of the pRT733(*TnphoA*) system in *B. mallei*. The optimised protocol did work; however, it was not considered efficient enough for our purposes. Although the frequency of PhoA+ mutants was 2 %, in practice, when a large number of matings were performed simultaneously (30), not all matings resulted in PhoA+ mutants. Whereas some matings would result in up to eight PhoA+ mutants, these mutants were considered to be identical. Some

PhoA+ mutants would also turn white and lose PhoA activity, which suggested instability of the transposon and/or PhoA fusion proteins. The isolation of flanking clones was found to be an extremely inefficient process. Approximately 10 % or less of the mutants resulted in the isolation of flanking clones. The isolation of flanking clones involved digesting the chromosomal DNA isolated from the PhoA+ mutant with either SalI or BamHI and ligating the resulting digest into the appropriately digested pBR322. This would result in an insert containing the *TnphoA* (7kb) and the DNA upstream of the *TnphoA* insertion. However, pBR322 has a limit of the size of DNA that can be efficiently inserted into it of approximately 7 kb (the size of the *TnphoA*). Therefore, the combination of limited restriction sites and the large size of the *TnphoA* probably contribute to the inefficiency of this system.

A manuscript describing the studies detailed above is attached as Appendix 2.

Development of an Alternative Transposon Delivered Phoa Fusion System

The transposon system, which appeared to have the most potential, was the minitransposon self-cloning vector *Tn5mod-OGm*. Minitransposons are specialized transposons which arrange the cognate transposase outside of the transposon's inverted repeats. This arrangement allows the minitransposon to integrate into target DNA without its transposase and prevents further transposition and DNA rearrangements. These synthetic transposons are small and stable and exhibit virtually no preference for specific target DNA sequence (unlike the *TnphoA* system). The *Tn5Mod-0Gm* is constructed with a conditional origin of replication within the transposon and allows the rapid cloning of the DNA adjacent to the transposon's site of insertion. Furthermore, rare restriction endonuclease sites are incorporated near the inverted repeats containing a total of 18 restriction sites. Analysis of the pmini-*Tn5Mod-OGm* DNA sequence indicated that insertion of the *phoA* gene without a signal sequence into the last restriction site (*KpnI*) of one of the multiple cloning sites in the correct orientation could result in a fusion protein when the transposon inserted into the recipient chromosome. As a result of the insertion of *phoA* into the pmini-*Tn5Mod-OGm*, two restriction sites would no longer be available for cloning (*DraI* and *KpnI*) due to there presence in the *phoA* insert; however, sixteen cloning sites would still be available.

Preliminary mutagenesis experiments with GB8 and *Tn5Mod-OGm* resulted in approximately 1500 transconjugates per mating, a frequency 10-fold higher than the pRT733(*TnphoA*).

Construction of *phoAMod-OGm*. Primers were constructed containing *KpnI* sites to the alkaline phosphatase gene (*phoA*) of *E. coli* (without the signal sequence). The *phoA* gene was amplified using PCR with pRT733 (*TnphoA*) as the template. The resulting PCR fragment and the pmini-*Tn5Mod-OGm* were digested with *KpnI* and the vector was dephosphorylated with CIP. Following ligation and subsequent transformation into *E. coli* DH5α, 9/50 transformants contained the *phoA* gene. Digestion of the plasmids with *SphI* determined that 3 plasmids contained the *phoA* gene in the correct orientation. The resulting plasmid is preliminarily named pmini-*Tn5phoAMod-OGm*. pmini-*Tn5phoAMod-OGm* was subsequently transformed into *E.coli* SM10.

***TnphoA* mutagenesis utilising pmini-*Tn5phoAMod-OGm*.** Transfer of *phoA-Mod-0Gm* into G8PN utilised the protocol developed for pRT733(*TnphoA*) with the exception that gentamicin (5ug/ml) was incorporated into the selection agar instead of kanamycin. Preliminary experiments resulted in approximately 1000 to 2000 transconjugates per mating, with a frequency of PhoA+ mutants of 1.5 %. Flanking clones have been isolated from the preliminary mutagenesis experiment with a success rate of 100% (6/6). At present, plasmids are being isolated, and sequencing will determine the effectiveness of this system. However, results appear promising because flanking clones possess PhoA activity indicating that they contain a phoA fusion protein. Therefore, preliminary experiments indicate that this system appears more efficient than the pRT733(*TnphoA*).

A manuscript describing the studies dtailed above is attached as Appendix 3.

Analysis of PhoA+ Mutants Derived From pRT733(*TnphoA*)

PhoA+ G8PN mutants were screened for loss of α-haemolytic activity and lipase activity. One mutant (AJB53) did not appear to possess lipase activity; sequencing of the *TnphoA* gene fusion is in process Computer searches of the DNA sequence isolated from the flanking clone of the PhoA+ mutant AJB34 indicated that the *TnphoA* had formed a fusion with a protein exhibiting high homology to the dipeptide transport system permease protein (DPPB) of *E. coli*. The search also indicated the presence of a second open reading frame immediately upstream from the DPPB homologue. The second orf exhibited high homology to an *E. coli* periplasmic dipeptide-binding protein (DBP), which also serves as a chemoreceptor in *E. coli*, and as a haemin-binding protein (HbpA) in *Haemophilus influenzae*. The *B. mallei* DBP/HbpA homologue will be further investigated.

Isolation of a putative two component response regulator OmpR/EnvZ

We previously determined that the *B. mallei* mutant AJB108 contains a *phoA* fusion in an unknown protein, however, upstream we located a putative homologue to the histidine kinase EnvZ. Predicting a homologue to the regulator OmpR upstream of the EnvZ homologue, we isolated a larger flanking clone and sequenced upstream of EnvZ. As a result we successfully identified an open reading frame with high homology to OmpR and RisA, regulators involved in intracellular survival of macrophages and invasion. We also identified these genes in *B. pseudomallei*. Allelelic exchange mutants are being constructed in *envZ* and *ompR* in *B. mallei* and *B. pseudomallei* and will be tested for loss of virulence and intracellular survival in macrophages.

Burkholderia mallei mini-O*phoA* mutants: screening for loss of virulence in hamsters

B. mallei mutants containing *phoA* fusions in genes showing homology to putative virulence associated or unknown genes were tested for loss of virulence in hamsters. We also tested the mutant AJBY2 which does not contain an inframe *phoA* fusion, however, sequence analysis indicated that the transposon had inserted within a probable polyketide synthase, because macrolides have been implicated as immunosuppressants this mutant was considered potentially interesting. Four mutants showed a reduction in virulence compared to the parent:

1. AJB108: insertion in an unknown protein downstream of the OmpR/EnvZ putative two component response regulator,
2. AJB138: insertion in a protein homologous to a putative integral membrane proteinase or regulator of a proteinase downstream from the virulence regulator HF-I,
3. AJB162: insertion in a protein with homology to an outer membrane lipoprotein which has been implicated in colonisation
4. AJBY2: a mutant with an insertion within a probable polyketide synthase.

We intend to determine the biological function of the mutated genes, but first we are focusing on the OmpR/EnvZ region and if AJBY2 is less toxic to immune cells.

Survival of the mini-OphoA *B. mallei* capsule mutant in J774 macrophages and horse blood

We had previously determined that both *B. mallei* and *B. pseudomallei* enter J774 macrophages and kill the cells following overnight incubation. We initially used the wildtype *B. pseudomallei* as a positive control but determined that effective antibiotic killing and inhibition of growth were difficult due to the high intrinsic resistance to antibiotics. Because the effectiveness of the antibiotic in this assay is crucial, we chose to use the *B. pseudomallei* antibiotic efflux pump deletion mutant DD503, which is susceptible to antibiotics such as kanamycin and gentamicin. This mutant is virulent in hamsters and we determined that it also kills J774 cells.

We also determined that because *B. mallei* is very susceptible to antibiotic killing, very low concentrations of antibiotic were needed for overnight incubation, 5 ug/ml, this is particularly important because organisms escape from the macrophages and are killed by the antibiotic if the concentration is too high, resulting in a deflated number of viable organisms.

Fig. 1

Strain	% 2h	% o/n
DD503	7.5	17.3
GB8	2.8	1
GB8(nhs)	4.2	1
G8PN(nhs)	4	1
AJB107(nhs)	2.3	0.16

Note: GB8=B. mallei
G8PN: parent (acid phosphatase mutant)
AJB107: B. mallei mini-O*phoA* capsule mutant.
(nhs): cultures were grown in 20 % normal human serum.
%: percentage of viable bacteria recovered relative to the initial inoculum.
2h: intracellular bacteria at 2 h post infection
o/n:intracellular and escaped bacteria following overnight incubation.

Using the optimised assay, we determined that although DD503 replicates within J774 cells two fold (Fig. 1), *B. mallei* does not, and shows a four fold reduction in viable bacteria following overnight incubation. However, it may be difficult to compare *B. mallei* and *B. pseudomallei*

because of the significant differences in growth rates. The *B. mallei* capsule mutant appeared to survive 6 fold less than the parent strain, a repeat experiment resulted in a 7 fold reduction in intraceullar survival.

We also performed a preliminary test of the survival of *B. mallei* and the *B. mallei* capsule mutant in fresh horse blood overnight. We diluted 1 ml of fresh whole horse blood with 1 ml of tissue culture medium and incubated with *B. mallei* 1 x 10^4 cfu/ml (this is the concentration of *B. mallei* organisms recovered from infected hamsters immediately prior to death). Fig. 2 indicates that *B.mallei* is not killed significantly by fresh horse blood, however, the capsule mutant is killed 100 fold.

Fig. 2

Strain	Inoculum	Cfu o/n	o/n killing
G8PN	$2x10^4$	$8x10^3$	2.5
AJB107	$2x10^4$	$2x10^2$	100

It has been difficult to obtain fresh horse blood, so future experiments will focus on human blood. We plan to look at the interaction of *B. mallei* and the capsule mutant with human neutrophils. Two possibilities which have been suggested as roles for the capsule in *Mycobacterium tuberculosis* are that it acts as a passive barrier by impeding the diffusion of bactericidal macromolecules into the bacterial cell. Toxic lipids and contact-dependant lytic substances, as well as constituents that inhibit both macrophage priming and lymphoproliferation, have been found in the capsule. It is possible that the *B. mallei* capsule protects the bacterium from the immune response in a similar fashion?

Construction of *gacA* allelic exchange mutants in *B. mallei*, *B. pseudomallei*, and a *B. pseudomallei* capsule:lacZ fusion mutant

The global activator GacA has been implicated in the positive regulation of a number of virulence determinants including secreted protease, lipase, toxin and capsule. We have constructed potential *gacA* mutants in *B. mallei*, *B. pseudomallei* and a *B. pseudomallei* capsule mutant which contains an in-frame lacZ fusion within the phosphomannomutase (SLR18). At present we have potential double crossover mutants in *B. pseudomallei* and the *B. pseudomallei* capsule mutant and we are selecting for a double crossover mutant in *B. mallei*.

Following confirmation of insertional inactivation of the *gacA* allele in *B. mallei* and *B. pseudomallei*, mutants will be tested for loss of virulence in the hamster model of glanders. The *B. pseudomallei* capsule mutant will be analysed for loss of capsule expression utilising a B-galactosidase assay in collaboration with Shauna Reckseidler. If the GacA homologue regulates capsule expression in *B. pseudomallei* we shall endeavour to contruct a lacZ fusion with the phosphomannomutase of *B. mallei* and perform similar expression assays. We shall also determine the affect GacA has on secreted products such as protease, lipase, phospholipase and haemolysin, and other virulence determinants it may regulate.

Construction and screening of plasmid expression libraries of *B. mallei* and *B. pseudomallei*

As mentioned above, GacA has been shown to regulate secreted products such as protease and lipase, *B. mallei* appears to secrete lipase, however, no protease activity has been shown to be secreted *in vitro*, however, it is not known if this occurs *in vivo*. To overcome possible expression controls we constructed plasmid expression libraries of partially digested genomic DNA (approximately 5 kb) of *B. mallei* and *B. pseudomallei*. Because *B. pseudomallei* is known to secretes protease(s) *in vitro*, we screened the *B. pseudomallei* library first to determine if this approach would successfully isolate proteases and lipases.
We screened 11, 000 recombinant clones and isolated 7 clones exhibiting protease activity on skim milk plates, plasmids have been isolated from three of the clones, and contain inserts sizes of 5, 6 and 7 kb, which are presently being sequenced.

We have also screened the *B. pseudomallei* library for lipase and phospholipase activity but no positive clones have been isolated as yet.

Analysis of the *B. mallei* plasmid library indicated insert sizes of 1 to 3 kb, which are too low to be representative, the *B. mallei* library shall be reconstructed.

Invasion and Intracellular Survival of *Burkholderia mallei*

Little is known about the virulence factors and pathogenesis of *B. mallei*, although the chronic and highly invasive forms of infection suggest that this pathogen possesses mechanisms for both cellular invasion and evasion of the host immune response. The objective of this work is to analyze the invasion and intracellular survival of *B. mallei* for human alveolar epithelial cell line (A549) and a human monocyte cell line (U937) which differentiates into macrophage-like cells when treated with phorbol 12-myristate 13-acetate (PMA).

A549 invasion assays. Invasion assays involved infecting confluent monolayers of eucaryotic cells (10^6 cells) with 10^7 organisms for 2 h at 37°C in 5 % CO_2. Samples were assayed in triplicate. Following a 1 h incubation with 150 ug/ml kanamycin and subsequent washes, cells were lysed with triton X-100. Intracellular bacteria were quantitated by plating serial dilution of the lysate. Routinely, the invasive *B. pseudomallei* strain 1026b was used as a positive control and the non-invasive *E. coli* HB101 was used as a negative control.

Preliminary experiments indicated that *B. mallei* strain GB8 only invaded A549 cells at low levels, 10 fold greater than *E. coli* HB101, and 100 fold less than 1026b. Cells did not appear to be killed by the bacteria as determined by a MTT assay. Centrifugation appeared to increase the recoveries of GB8 (a non-motile strain) but not 1026b (a motile strain). The growth conditions also appeared to affect invasion, for example, *B. mallei* grown in media supplemented with either 5 % normal human serum (NHS) or heat inactivated horse serum, appeared to be approximately 50 fold more invasive than grown without serum. However, it has been shown with other invasive pathogens that growth phase itself can affect the invasion of eucaryotic cells. To determine that the preliminary data was not due to growth phase alone, an invasion assay was performed using cultures of *E. coli* HB 101, *B. pseudomallei* 1026b and *B. mallei* GB8 at different phases of growth.

Table 1.

Strain	Growth Media	Growth Conditions (agitation/time)	Growth Phase	Mean % invasion	SD
HB101	LB	agitated (3h)	mid log	0.003	0.001
1026b	LB	agitated (3h)	mid log	7.069	3.02
GB8	TSAG	agitated (5h20')	mid log	0.033	0.005
GB8	TSAG+NHS	non-agitated (4h)	lag	1.217	0.151
GB8	TSAG+NHS	agitated (3h)	lag	1.263	0.169
GB8	TSAG+NHS	agitated (5h)	mid log	0.323	0.048
GB8	TSAG+NHS	agitated (6.5h)	late log	0.155	0.045
GB8	TSAG+NHS	agitated (o/n)	stationary	2.653	0.468

Note:
TSAG: Tryptic soy broth supplemented with 4 % glycerol.

NHS: Normal human serum (final concentration 5%)

Table 1 shows that all cultures of GB8 supplemented with NHS were more invasive than TSAG alone. This may represent that *B. mallei* contains a receptor for a serum component that acts as a signal transducer (switching on genes involved in invasion). Alternatively, acquisition of a serum factor by *B. mallei* may be required to attach to epithelial cells prior to invasion (as is found with the fibronectin binding protein of *Streptococcus pyogenes*). Unexpectedly, it appeared that lag and stationary cultures were more invasive than the log phase cultures; active growth appears to diminish invasion, an observation that is in contrast to other pathogens. The non-agitated culture (oxygen limited) appeared to be more invasive than the agitated log cultures; this finding is consistent with the invasion of Salmonella, where growth of this pathogen under oxygen limited conditions promotes invasion.

Intracellular survival of B. mallei within the U937 cell line. GB8 was grown under different growth conditions and differentiated U937 cells (10^6 cells) were infected with 10^7 organisms for 2 h at 37°C in 5 % CO_2. Following a 1 h incubation with 150 ug/ml kanamycin and subsequent washes, cells were either lysed with triton X-100 (Time 0) or incubated for 18 h with fresh media containing 30ug/ml kanamycin (time 18h). Intracellular bacteria were quantitated by plating

serial dilution of the lysate. Routinely, the invasive *B. pseudomallei* strain 1026b was used as a positive control and the non-invasive *E. coli* HB101 was used as a negative control.

Table 2.

Strain	Growth Media	% inv Time 0	% inv 18 h
HB101	LB	12.5	8.19
1026b	LB	14.87	65.5
GB8	TSBG-DC	1.63	0.14
GB8	TSAG+NHS	0.91	0.06
GB8	TSAG+HS	1.72	0.1
GB8	TSAG	0.77	0.07

Note:
LB: Luria broth
TSBG-DC: Tryptic soy broth supplemented with 4 % glycerol and chelated with Chelex-100 (6 h), dialysed overnight, autoclaved and supplemented with 0.05M monosodium glutamate.
TSAG: Tryptic soy broth supplemented with 4 % glycerol
HS: heat inactivated horse serum (final concentration of 5 %)
NHS: Normal human serum (final concentration of 5 %)
% inv: % invasion relative to the initial inoculum.

Low numbers of viable GB8 were recovered from U937 cells at time 0, and over 18 h, a log decrease in the number of viable organisms located intracellulary occurred. The growth conditions did not appear to affect intracellular survival of GB8. However, the negative control, HB101 was not killed efficiently, indicating these cells are not very bactericidal. A similar result has been observed with the J774 cell line. These cells should efficiently kill HB101. One possibility is that most researchers supplement the tissue culture medium with fetal calf serum (fcs), however, we have been using a cheaper product 'Fetal Clone', a bovine serum product which is not actually fcs. This may affect the bactericidal properties of these cells. If these cells are defective in the bactericidal pathway, these results could suggest that GB8 resists phagocytosis and/or escapes from the macrophage once ingested. Also another possibility is that GB8 may kill the U937 cells. Experiments designed to test each of these potential scenarios will be performed.

ATPase and NDK Production By *Burkholderia Spp.*

Intracellular pathogens such as *B. pseudomallei* has been shown to survive inside phagocytic cells rendering it able to evade the humoral immune response and the specific cell-mediated

response. How this organism survives inside the macrophage is not well documented. Studies of other *Burkholderia* spp. are also limited, especially for *B. mallei*. Studies from *Mycobacterium tuberculosis*, *M. bovis*, and other less virulent mycobacterium strains have shown that virulent strains can survive intracellularly, while non-virulent ones cannot. Recently, a new mechanism for killing of intracellular mycobacteria by apoptosis has been shown. This mechanism involves an activation of specific receptors, called P2Z, on the macrophage cell membrane by extracellular ATP. The activated receptors trigger the macrophages to go into programmed cell death, or apoptosis, and the intracellular bacteria die with the macrophages. The difference between virulent and avirulent mycobacteria has also been elucidated recently in that the secretion of exoenzymes, such as ATPase and NDK, appear to promote survival inside of macrophages. Upon production of ATPase or NDK, the extracellular ATP is used up, and the macrophages cannot go on to apoptosis; hence, virulent mycobacteria will survive in those macrophages. To assess any similarity between *Mycobacterium* and *Burkholderia*, ATPase, NDK, and cytotoxicity assays were performed in four different species of *Burkholderia*. The conclusions from our studies are as follows:

1. ATPase activity is present in the cell supernatant fluids from all *Burkholderia* spp. However, the ATPase in the supernatant obtained from E264 released radioactive ^{32}P, Pi, which is different from Pi* formed by ATPase extracted from GB8, 1026b, and K56-2. Also, E264 did not show any UTP, CTP, or GTP production indicating that there was no NDK activity.
2. The production of ATPase from E264 may be responsible for the increase in macrophage survival upon apoptosis stimulation by ATP.
3. The concentrated culture supernatants from GB8, 1026b, and K56-2 did not increase the survival rate of macrophages after they had been stimulated with LPS and ATP.
4. It remains to be seen there is any correlation between intracellular survival and an inhibition of the apoptosis in E264. Unlike *Mycobacterium bovis* BCG, avirulent *Burkholderia thailandensis* prevents apoptosis upon ATP activation, but the successful inhibition of macrophage apoptosis may not be involved in *Burkholderia* survival.
5. The preliminary results obtained here did not demonstrate any obvious differences among *Burkholderia mallei* GB8, *B. pseudomallei* 1026b, and *B. cepacia* K56-2.

Immunological Studies of *Burkholderia mallei* Infection in Syrian Hamsters and Major Histocompatibility Complex - Gene Knock Out C57BL/6 Mice

Introduction

B. mallei infection in golden Syrian hamsters always leads to acute infection and death, usually within 5 days, before any specific immune response can be measured or detected. This observation indicates that an innate response, such as the phagocytic activity from polymorphonuclear cells, complement, bactericidal peptides, etc. cannot kill all the bacteria. It is difficult to predict whether the hamster would be able to mount protective immunity against a *B. mallei* infection if the hamster could survive long enough to produce specific immunity. The hamster can however be challenged with *B. mallei* antigens without causing a lethal infection. One can use formalin-killed bacteria as immunogens to study the specific immune response 7-10 days after the challenge. As *B. mallei* is a facultative intracellular organism, and evidence exists to demonstrate that the humoral response might not be completely protective against *B. mallei* infections, we have begun to focus on the cell-mediated response in animal models.

Cell-mediated immune responses are dependent upon different sets of T cells. The cytotoxic response is dependent upon CD8+ cytotoxic T cells, which require the MHC class I molecule for antigen presentation. The cell-mediated immune response dependent upon CD4+ delayed-type hypersensivity T cells (T_{DTH}), requires the MHC class II. It is possible that *B. mallei* can invade and survive inside certain host cells, e.g. epithelial cells. Cytotoxic T cells can only kill infected cells, but not the bacteria. As a result, the intracellular bacteria are released to the extracellular compartment. However, the cytotoxic activity probably plays a significant role in eliminating the hiding places for these bacteria, and the professional phagocytic cells are provided an opportunity to remove the extracellular bacteria. The professional phagocytic cells that have the best chance to kill *B.* mallei are likely to be activated macrophages, which are effector cells of T_{DTH}.

Macrophages require γ-interferon (γ-IFN) from T_{DTH} to be activated, and macrophage activation results in a delayed type hypersensitivity (DTH) response. The goals of the present studies were to measure the DTH response in susceptible animals challenged with *B. mallei* antigens, and to determine whether the hamsters survived a subsequent challenge with wild type *B. mallei* after the specific immune response had been produced. To accomplish these goals, we developed the following specific aims:

1. To determine whether specific immunity improves the outcome of *B. mallei* infection.
2. To demonstrate the role of the specific cell-mediated response via activated macrophages in *B. mallei* infection in susceptible animals.

Materials and Methods

Syrian hamsters model. To determine whether hamsters were able to produce a specific immune response against *B. mallei* the following experiment was performed. Hamsters were divided into 3 groups of 3 animals; the first group served as a control and was treated with PBS; the second group was treated with 5 % formalin-killed *B. mallei* strain GB8; and the third group

was treated with tuberculin solution. The DTH response was determined at two different sites: the subcutaneous injection site of the abdomen and the hind foot pad. Finally, the hamsters were challenged with a lethal dose of GB8 (approximately 2000 CFU), and the progression of the disease in the different groups of hamsters was compared.

DTH response. Hamsters were challenged with 5 % formalin-killed whole bacterial cells. *B. mallei* GB8 were grown over night to about 10^8 CFU/ml. The cells were harvested and resuspended in 5 % formalin in PBS for 30 minutes. The cells were centrifuged and washed three times with PBS. The final pellet was resuspended in 2 ml PBS, to a density of about 5 $\times 10^8$ cells/ml. The initial challenge dose was 50 µl of the cell suspension injected subcutaneously at each of the lower two quadrants of the abdomen. The DTH response was determined at two weeks after the initial challenge by injecting 50 µl of the 5 % formalin-killed cell suspension subcutaneously into the upper two quadrants of the abdomen and also in the left hind foot pad. The diameter of the induration at the abdomen and foot pad thickness were determined at 24 hr after the second injection.

MHC gene knock out mouse model. Different strains of MHC knock out (C57BL/6) mice obtained from Taconic were used to determine the type of cell-mediated response most important in protection against *B. mallei* infection. The MHC class I knock out strain, β_2m gene knock out, lacks class I MHC molecules that are necessary for CD8+ cytotoxic activity. The MHC class II knock out strain, Abb gene knock out, lacks class II MHC molecules that are necessary for CD4+ T cell function. The double MHC class I and II knock out, which lacks both classes of MHC molecules, served as a control. There were two groups of two mice per strain; the first group was challenged with *B. mallei* ~10^4 CFU; the second group was challenged with ~10^5 CFU.

Results

Lethal dose challenge in hamsters after stimulation with 5 % formalin-killed *B. mallei* GB8. Two hamsters from each group (PBS, 5 % formalin-killed cell, and tuberculin treated) were challenged intraperitoneally with ~2,000 CFU of *B. mallei* GB8 that had been passaged through hamsters three times. All hamsters from the PBS and tuberculin-treated groups were dead on day three, while the two hamsters in the 5 % formalin-killed cell treated group were still alive. These two hamsters died on day four.

DTH response in hamsters.

1) Primary challenge.

- PBS-injected group: The group injected with PBS showed no sign of inflammation at the PBS injection sites.
- 5% formalin killed GB8 group: All of the hamsters injected with 5 % formalin-killed GB8 showed some degree of inflammation as indicated by red lesions of approximately 3-4 mm induration at the injection sites 24 hr after the injection.
- Tuberculin-injected group: One of the hamsters injected with tuberculin solution showed about 1 mm induration at the site of injection, but the other two showed no signs of inflammation.

2) Secondary challenge. The induration at the abdominal sites was larger than that seen after primary challenge in the 5 % formalin-killed cell injected group, and the groups injected with PBS or tuberculin did not show any induration. A change in foot pad thickness change also occurred in the 5 % formalin-killed cell injected group and not in the PBS or tuberculin injected groups. Table 1 shows the size of induration at the abdominal injected sites and the changes in foot pad thickness at 24 hr post secondary challenge. The results at 48 and 72 hr post secondary challenge were similar to those noted at 24 hr in all animals.

Table 1. The DTH response to *B. mallei* GB8 in hamster.

		Abdomen	Hind foot pads		
	number	induration diameter (mm.)	Left foot (mm.)	Right foot (mm.)	% thickness change
PBS group	1	0	3	3	100
	2	0	3.05	2.9	105
	3	0	2.8	2.85	98
5% formalin killed GB8 group	1	9.7	5.3	2.9	183
	2	7.4	4.9	2.9	169
	3	8.4	4.9	2.9	169
Tuberculin group	1	0	2.9	2.9	100
	2	0	3.1	3.1	100
	3	0	3.2	2.9	110

Susceptibility of class I MHC gene knock out C57BL/6 mice. The mice in all groups of class I MHC knockout mice survived for two weeks. At the end of the second week, one mouse from each group was sacrificed, and internal organs were examined. The spleen, liver, and lungs were harvested for bacterial culture and PCR analysis using primers specific for *B. mallei* 16S ribosomal DNA. The gross pathology of all organs were normal, the bacterial cultures showed no growth, and PCR anslyses were negative for *B. mallei* DNA in all animals.

Susceptibility of class II MHC and double MHC gene knock out mice. Two months after *B. mallei* GB8 challenge, the last mouse in each group was sacrificed, the internal organs were examined and bacterial cultures were performed on these. PCR analysis is in progress. The class II knock out mouse showed spleen enlargement, approximately 4 times larger than normal, with miliary nodules covering the spleen. The liver was approximately 1.5 times larger than normal. The double gene knock out mouse also showed spleen and liver enlargement to the same degree as in the class II knock out mouse. Furthermore, the double gene knock out mouse demonstrated signs of infection in the pleural cavities, as there was blood collected in both sides of the pleural cavity. Bacterial culture of homogenized organs of these two knock out mice, class II and double knock out, resulted in bacterial growth on LB agar plates characteristic of *B. mallei.*

Summary

Formalin-treated whole bacterial cells do protect against lethal infection in the hamster model. This failure may be due to the inability of the killed bacteria to up-regulate the cell-mediated response. It is most likely that the hamsters can mount specific immune response to *B. mallei*,

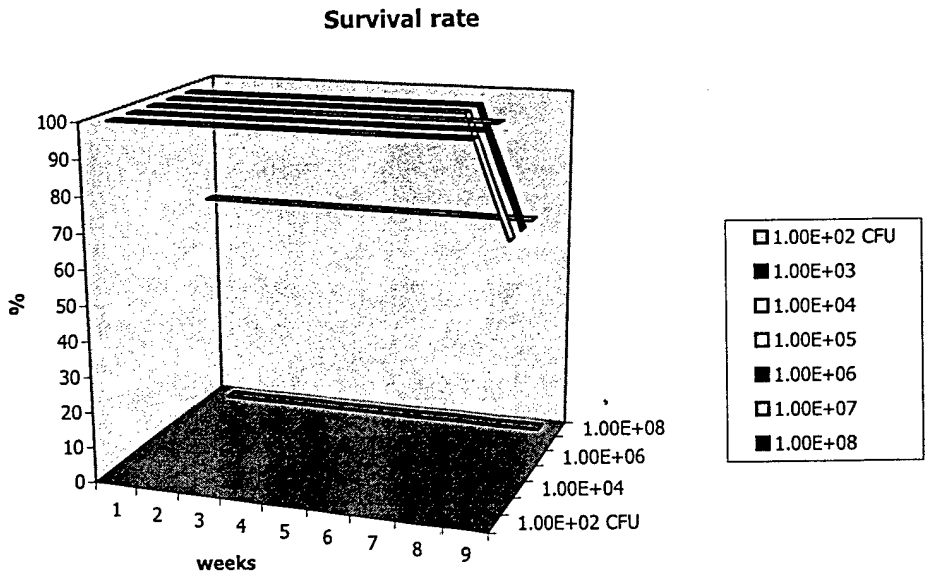
but the phagocytic cells, such as activated macrophages, were not highly responsive, or the bacterial killing mechanism is defective in hamsters (this also includes the defect in polymorphonuclear cell killing activity).

The hamster clearly produces a DTH response to *B. mallei* antigens, as shown by an increase in the size of the indurations at the sites of secondary antigen challenge and the increase in foot pad thickness. In future experiments we will use separate, purified antigens, and the dose will be adjusted, so that the degree of DTH stimulation can be measured for each separate antigen. We expect that particular antigens will best serve to specifically enhance the DTH response.

We are very excited about using the class II MHC gene knock out mice as an animal model for chronic *B. mallei* infections. These animals will be of significant practical use in our immunological studies due to the availability of mouse reagents for use in these types of studies.

Chronic *B. Mallei* Infection In Wild Type Mice

The findings from *B. mallei* GB8 infected C57BL/6 (Taconic) mice showed that they could be chronically infected. Animals that were challenged with > 10^6 CFUs developed splenomegaly, became ill around the end of first month, and died about the end of second month. The enlarged spleen obtained from the animals showed multiple abscesses with caseous necrosis, and spleen cultures showed positive growth for *B. mallei* GB8 organisms. The spleens that were in normal size range have not yet been tested for the presence of the organisms.



Survival rate of C57BL/6 mice after different inoculum sizes of GB8 challenged intraperitoneally.

Table 2. Spleen size of C57BL/6 mice challenged with GB8 at the end of week 10.

group	animal #1	#2	#3
10^2 CFU	0*	0	0
10^3	++	0	0

10^4	0	0	0
10^5	++	++	0
10^6	+++	++	++
10^7	0, acute**	0, acute	++
10^8	0, acute	0, acute	0, acute

* 0, normal spleen size; ++, 5 times larger; +++,10 times larger (approximation). ** acute, the animal died within one week after the challenge.

In conclusion, these findings have shown that the wild type mouse (C57BL/6) can be used as a model for chronic *B. mallei* infection. This strain can also be used in studies such as active immunization, effects of antibiotics, bacterial survival, etc. We do not need MHC knock out mice which need complicated cellular transfer to make them suitable for active immunization study. The mouse model should facilitate vaccine development, because the reagents for mice can be obtained readily.

Aminoglycoside And Macrolide Susceptibility In *Burkholderia Mallei*

Unlike *Burkholderia pseudomallei*, *B. mallei* is sensitive to aminoglycoside antibiotics such as streptomycin, gentamycin and tobramycin and macrolide antibiotics such as azithromycin and clarithromycin. Resistance to both aminoglycosides and macrolides in *B. pseudomallei* is due to an efflux-mediated system. Mutants of *B. pseudomallei* lacking the efflux system display an 8 - 128 fold increase in aminoglycoside susceptibility. We were interested in determining whether *B. mallei* was susceptible to aminoglycoside and macrolides due to loss of efflux genes. Southern hybridizations were performed using an internal region of the efflux operon gene *amrA* from *B. pseudomallei* as a probe for homologous *amrA* sequences in *B. mallei* chromosomal DNA. Hybridization results indicated the presence of homologous *amrA* sequences in six of nine *B. mallei* strains examined suggesting the presence of an efflux system in these strains. We are currently trying to complement these strains with cloned regions of the *amr* locus from *B. pseudomallei* in order to determine if *B. mallei* lacks certain components of the efflux system.

Studies on the β-lactamases from *Burkholderia* spp.

Aim of study: To identify the DNA sequences of β-lactamase genes from *Burkholderia mallei*, *B. pseudomallei*, and other closely related species.

Materials and methods

1. Direct cloning: The chromosomal DNA from *B. cepacia*, *B. thailandensis*, *B. mallei*, and *B. pseudomallei* were extracted and digested with various restriction enzymes, then religated to pUCP24 plasmids, which had been digested with relevant enzymes. The recombinant plasmids were used in electroporation or transformation of *E. coli* DH5α, and the transformants were selected on LB agar plates with 30 μg/ml ampicillin or 8 μg/ml cefazolin.

2. PCR for β-lactamase genes: The β-lactamase gene sequence of *B. cepacia* 249 strain was obtained from GenBank, and used as a template for primer selection. The sequences of primers are followed;
   PenA forward: 5'-AGCGCTCAATTATCGTTGCTTG-3'
   PenA reverse: 5'-GCGTCTGCGTGTAGTAGACGG-3'
   SDN1: 5'-CAGCGACAACAC-3' (based on 249 strain)
   SDN2: 5'-AGTACAGCGACAAT-3' (based on K56-2 strain)
   KTG: 5'-GCCSGTCTTGTC-3' (S = C or G)
   The PCR thermal cycler was set to run at 97 °C for 5 minutes; 95 °C 1 minute, 32 or 34 °C for 30 seconds, 72 °C for 30 seconds, 30 cycles; 72 °C for 10 minutes; then 4 °C on hold.
3. TOPO cloning using PCR products: The PCR products were removed from the agarose gel, and extracted for direct TOPO cloning (Invitrogen) using the protocol according to the manufacturer.

Results

1. Direct cloning: Numerous attemps to select ampicillin or cefazolin resistant transformants were unsuccessful.

2. PCR for β-lactamase genes:
   2.1 PCR using primers designed from GenBank database. The chromosomal DNA of *B. cepacia* K56-2 was used in the PCR with the first set of primers, PenA forward and reverse, which were designed from *B. cepacia* 249 strain (figure 1).

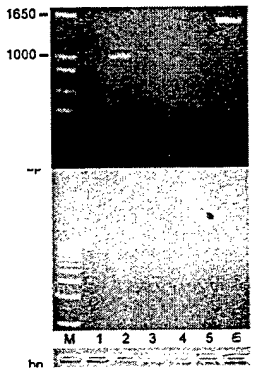


Figure 1. PCR using PenA forward and reverse primers. Lane 1, negative control using *B. cepacia* 249-2 DNA; lane 2, K56-2 DNA; lane 3, GB8 DNA; lane 4, E264 DNA; lane 5, 1026b DNA as templates, respectively. Lane 6, positive control for PCR condition using 16S-5' and 16S-3' rDNA primers and GB8 DNA as a template.

There was 1-kb DNA product amplified from chromosomal DNA of *B. cepacia* K56-2. There also was a faint band in the lane with *B. thailandensis* E264 DNA as a template, however the sequencing data was shown to be rod-shape determining gene and not β-lactamase gene. The

K56-2 PCR product was subsequently cloned, and the DNA sequence analysis revealed a strong homology to a *B. cepacia* 249 β-lactamase gene. Although the β-lactamase gene from *B. cepacia* K56-2 was not complete, only about 20 base pairs at the end of the gene were not obtained. The DNA sequence identity between *B. cepacia* K56-2 and 249 genes is about 79 %. The PCR products from *B. cepacia* K56-2 strain was later used in Southern blot analysis.

2.2 PCR using primers for consensus DNA sequence encoding the conserve SDN and KTG motifs of class A β-lactamase. Due to short sequences of the SDN1, SDN2, and KTG primers, the annealing temperature has to be low, about 32-34 °C, resulting in multiple products. However, the expected product from class A β-lactamase gene is about 320 bp, other products that have different size would not have much interference. One of the PCR results is shown in figure 2.

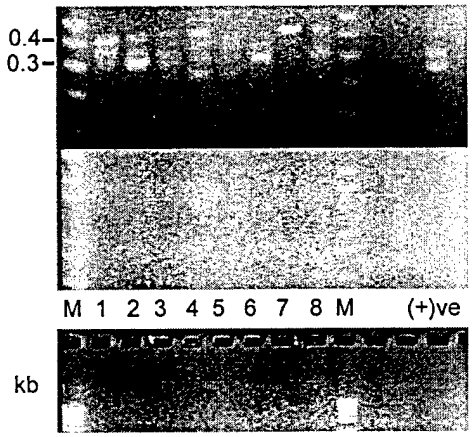


Figure 2. PCR products using SDN2-KTG primers. Lane 1-4 are chromosomal DNA digested with *Bam*HI, lane 5-8 are chromosomal DNA digested with *Eco*RI. Lane 1and 5, K56-2 DNA; lane 2 and 6, E264 DNA; lane 3 and 7, GB8 DNA; lane 4 and 8, 1026b DNA as templates. M, 1 Kb plus ladders. (+)ve, positive control using 1 kb fragment of K56-2 β-lactamase gene as a template.

3. Southern blot analysis:

3.1 The K56-2 β-lactamase gene was used as a probe to identify β-lactamase genes in other *Burkholderia* spp. As shown in figure 3, there were positive signals from E264, GB8, and also 1026b strains. Although the signal from 1026b was faint, due to low DNA amounts loaded on the gel, other experiments showed that K56-2 β-lactamase probe also hybridized 1026b DNA if more DNA was loaded (data not shown).

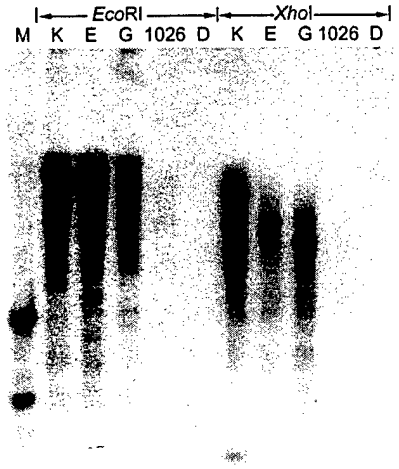


Figure 3. Southern blot analysis using 1kb fragment of K56-2 β-lactamase gene as a probe. M, DNA ladder; K, K56-2 DNA; E, E264 DNA; G, GB8 DNA; 1026, 1026b DNA as templates. All DNA were chromosomal DNA digested with *Eco*RI or *Xho*I.

3.2 The 326 bp segment of K56-2 β-lactamase gene was used as a probe. The PCR products from 4 different strains using SDNs-KTG primers were subjected to hybridization with shorter probe, 326 bp, in order to eliminate possible non-specific hybridization. The PCR products of expected size from E264, GB8, and 1026b gave positive signals as shown in figure 4.

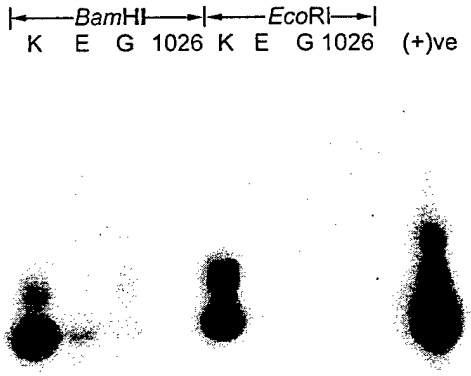
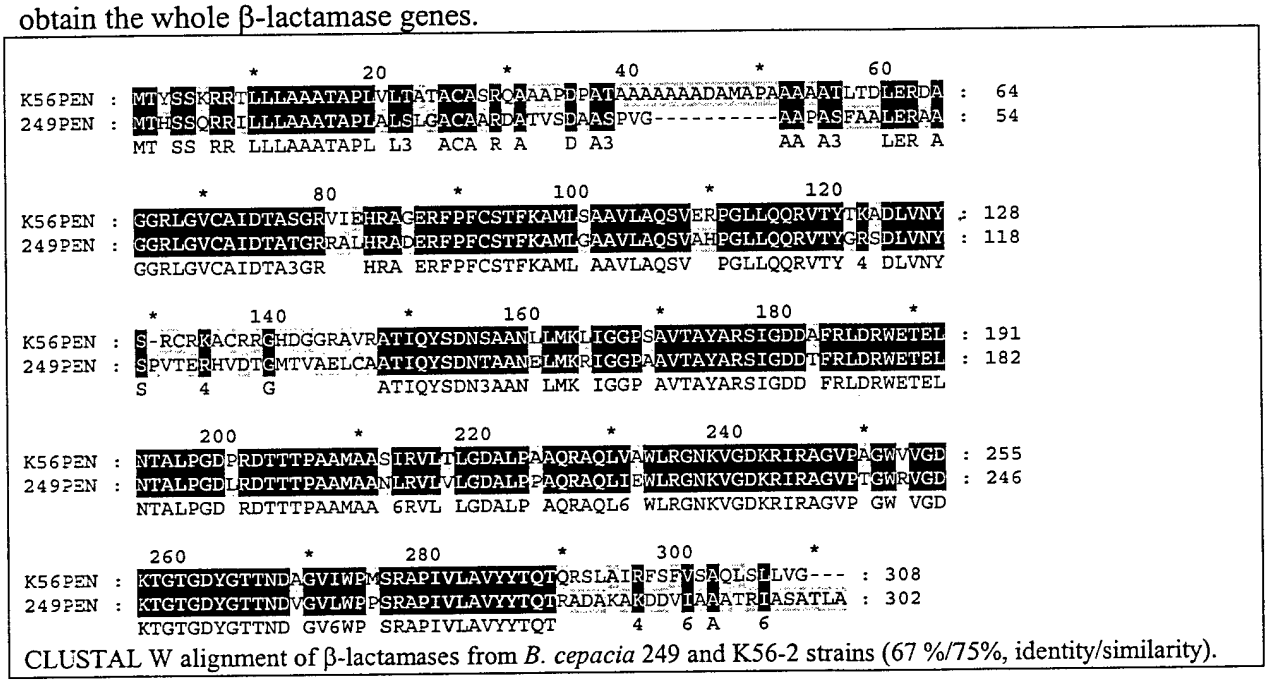


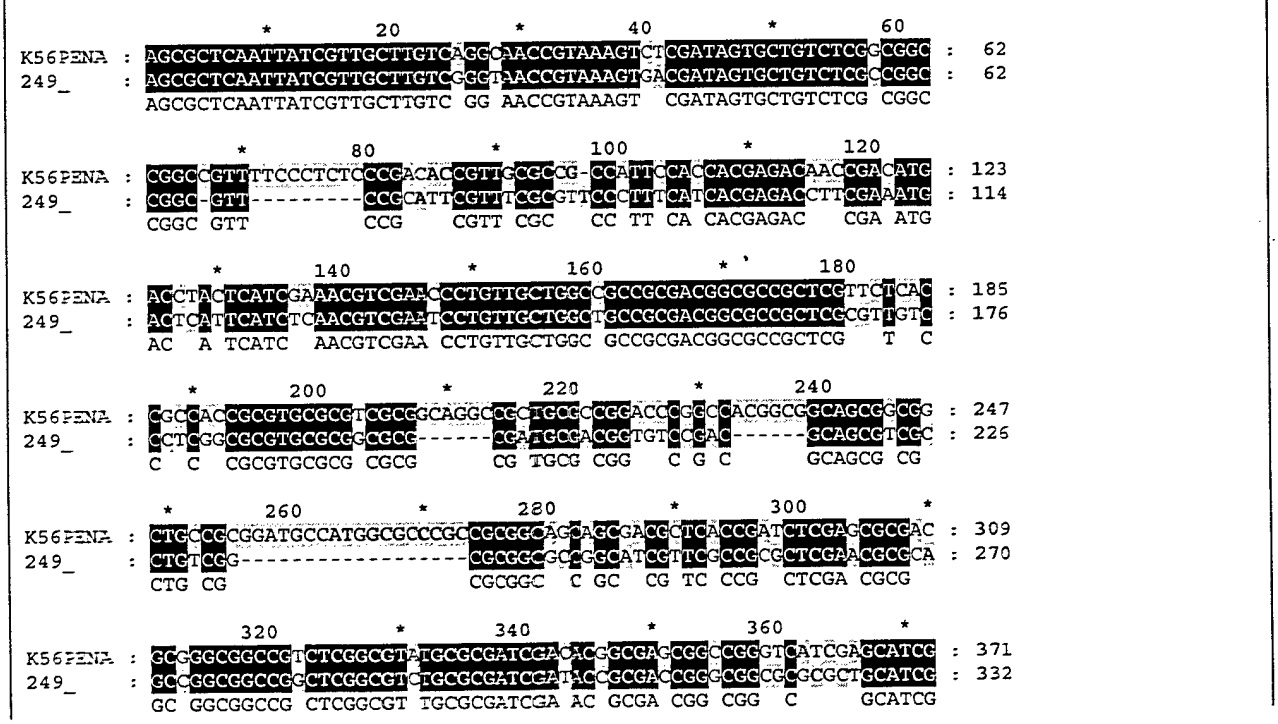
Figure 4. Southern blot analysis using 326 bp DNA fragment of K56-2 β-lactamase gene as a probe. The DNA for hybridization were PCR products amplified by using SDN2-KTG primers and *Bam*HI or *Eco*RI digested chromosomal DNA as templates. K, K56-2; E, E264 ; G, GB8; 1026, 1026b. (+)ve, positive control using 326 bp from K56-2 β-lactamase gene as template.

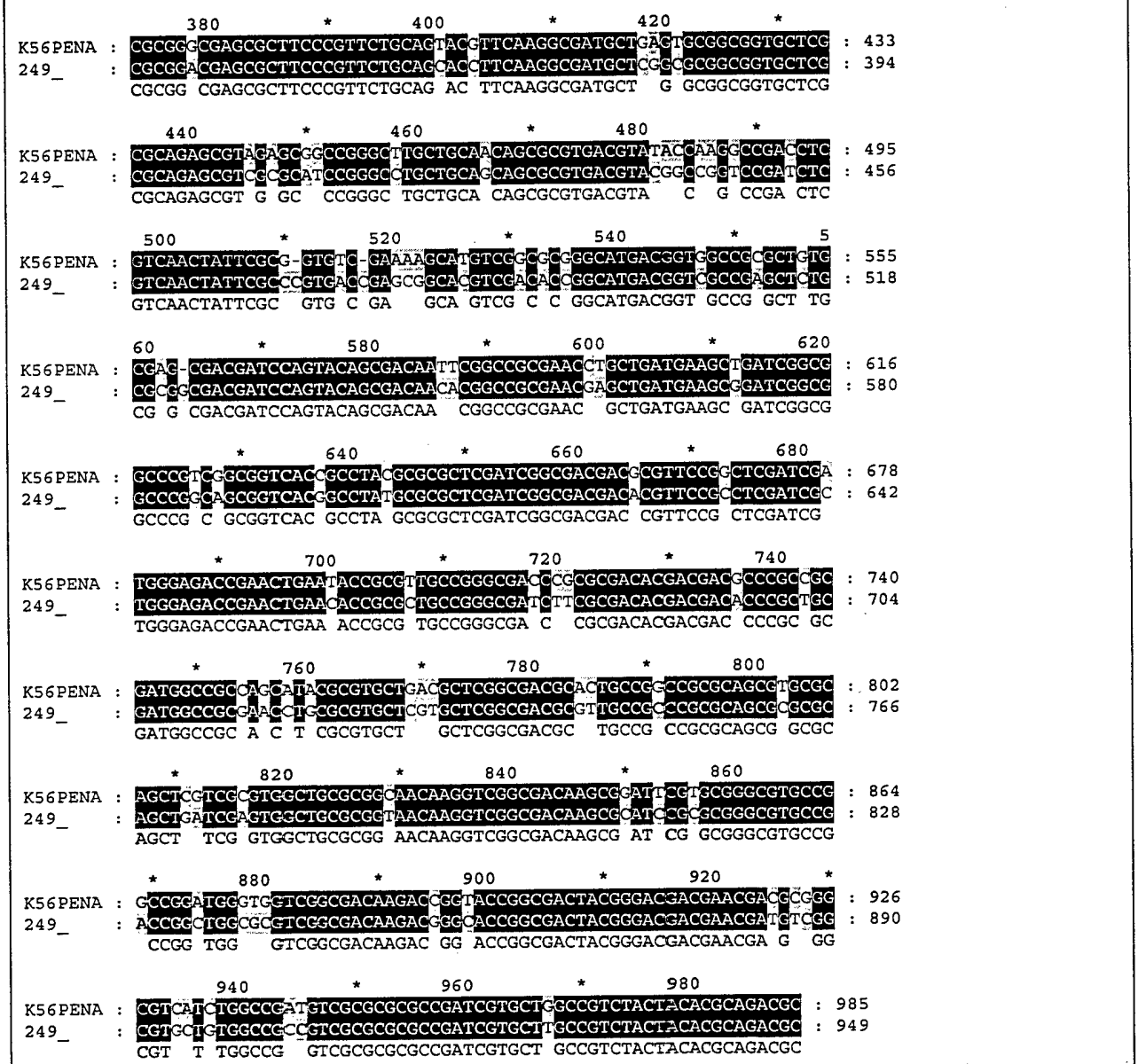
The production of β-lactamase in *Burkholderia* is one mechanism contributing to β-lactam resistance. According to previous studies, it may be postulated that the β-lactamase from *B. pseudomallei* and other related organisms, such as *B. mallei* and *B. thailandensis*, belong to class A β-lactamase. Unfortunately, the direct cloning experiment was unsuccessful; perhaps due to the lack of a suitable host for β-lactamase expression. Attempts to obtain β-lactam susceptible mutants using transposon mutagenesis were also unsuccessful. The PCR using primers designed from consensus segments of β-lactamase gene seems to be new and reasonable approach. GenBank data has shown that most of the high GC content organisms, such as *B. cepacia*, *Streptomyces*, *Nocardia* spp., have chromosomal class A β-lactamases, and almost all have

conserve motifs SDN and KTG in their β-lactamase sequences. However, the primers obtained from the consensus sequences are fairly short, and the amplified products may not be very specific. Attempts to perform direct cloning of PCR products with size of 320 bp yielded non-β-lactamase genes. The Southern blot analysis was adopted to specify which DNA fragments contain the correct β-lactamase gene segment. The screening of PCR products that hybridize with K56-2 β-lactamase probe is underway. Once the correct fragments are identified from *B. mallei*, *B. pseudomallei*, or *B. thailandensis*, these fragments will be used for library screening to obtain the whole β-lactamase genes.

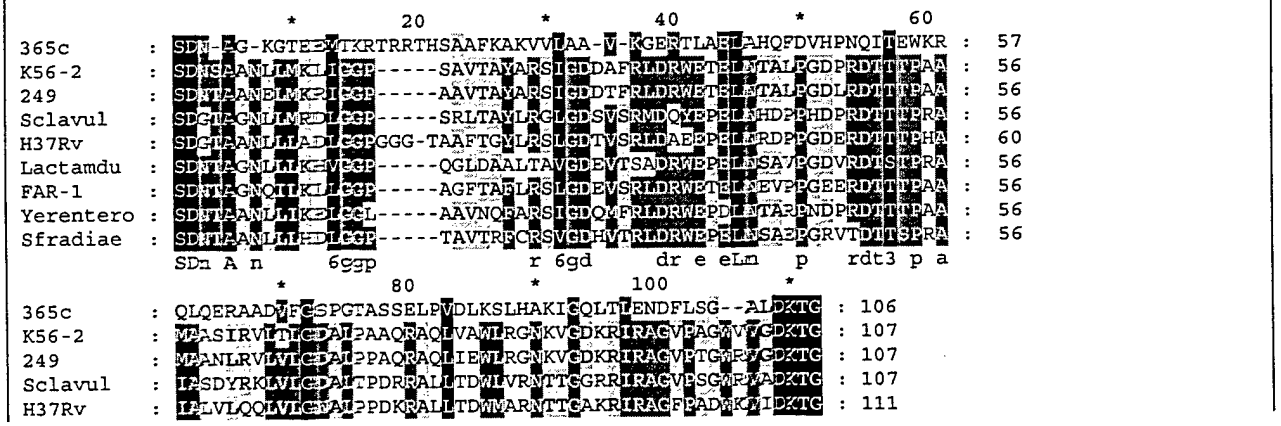


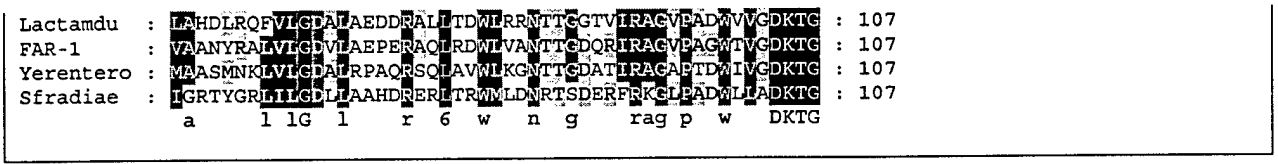
CLUSTAL W alignment of β-lactamases from *B. cepacia* 249 and K56-2 strains (67 %/75%, identity/similarity).





CLUSTAL W alignment of β-lactamase genes from *B. cepacia* 249 and K56-2 strains (79% identity).





CLUSTAL W alignment of SDN-KTG segments from different organisms that have class A β-lactamase genes. 365c, possible β-lactamase gene from *B. pseudomallei* 365c; K56-2 and 249, *B. cepacia*; Sclavul, *Streptomyces clavuligerus*; H37Rv, *M. tuberculosis* H37Rv; Lactamdu, *Nocardia lactamdurans*; FAR-1, *Nocardia farcinica*

Mini-OphoA insertions resulting in enhanced susceptibility to ß-lactams in *B. mallei* GB8.

Approximately 50 mini-OphoA *B. mallei* mutants were screened for altered sensitivities to ß-lactam antibiotics. Two mutants, AJB161 and AJB176, were found to have a significantly increased susceptibility to pipericillin and carbenicillin. Further testing demonstrated that the mutants had increased susceptibility to ceftazidime, ampicillin, amoxicillin and penicillin (Table 1).

Table 1. ß-lactam MICs of *B.mallei* mini-OphoA mutants

MIC ug/ml

Strain	pen	carb	amox	pip	ceft
GB8PN	>256	1024	>256	64	0.5
AJBY2	>256	1024	ND	32	0.5
AJB176	8	<2.0	.125	<1.0	.064
AJB161	8	<2.0	.125	<1.0	.047

GB8PN- parent, AJBY2- random mini-OphoA mutant

The mutants were not altered in susceptibility to tetracycline, trimethoprim, streptomycin and chloramphenicol. DNA flanking the mini-OphoA insertion was cloned and sequenced. Blast search analysis revealed both mini-OphoA insertions were located in a region having strong homology to fusaric acid resistance genes from *B. cepacia*. In *B. cepacia*, five genes have been identified which are involved in resistance to the fungal toxin, fusaric acid. Mutations in any of the 5 genes result in fusaric acid sensitivity however it is not known whether these genes are

involved in ß-lactam susceptibility. Fusaric acid MICs were determined in AJB176 and AJB161 and were not found to be significantly different from the parent strain. The mutants did not demonstrate increased susceptibility to lysozme lysis nor did they demonstrate enhanced nitrocefin (a chromgenic ß-lactam) hydrolysis suggesting that the mutations in AJB176 and AJB161 did not result in altered outer membrane permeability causing enhanced susceptibility to ß-lactam antibiotics. Southern hybridization experiments have demonstrated fusaric gene homologs in *B. thailandensis* and *B. pseudomallei*. We are currently sequencing the entire fusaric acid resistance region in *B. mallei* GB8 and will construct mutants to confirm the role of these genes in ß-lactam susceptibility. Further studies will be aimed at determining how these genes are involved in ß-lactam susceptibility in *B. mallei*.

Additional Studies on Beta-lactamase in *Burkholderia spp.*

1. Construction of *penA* knockout mutants in *B. mallei* and *B. thailandensis*.
2. Construction of *penA* and *fusE* double mutants.
3. Beta-lactamase purification and functional study.
4. The chronic *B. mallei* infection in wild type mouse model.

1. The conjugative plasmid (pGSVpen580, figure 1) was constructed containing a 580 bp internal fragment of the *B. mallei penA* gene. The plasmid has been used successfully to disrupt the *penA* gene in *B. thailandensis* E264 and *B. mallei* GB8 via homologous recombination. The preliminary MICs for amoxicillin for these mutants and wild type are shown in table 1.

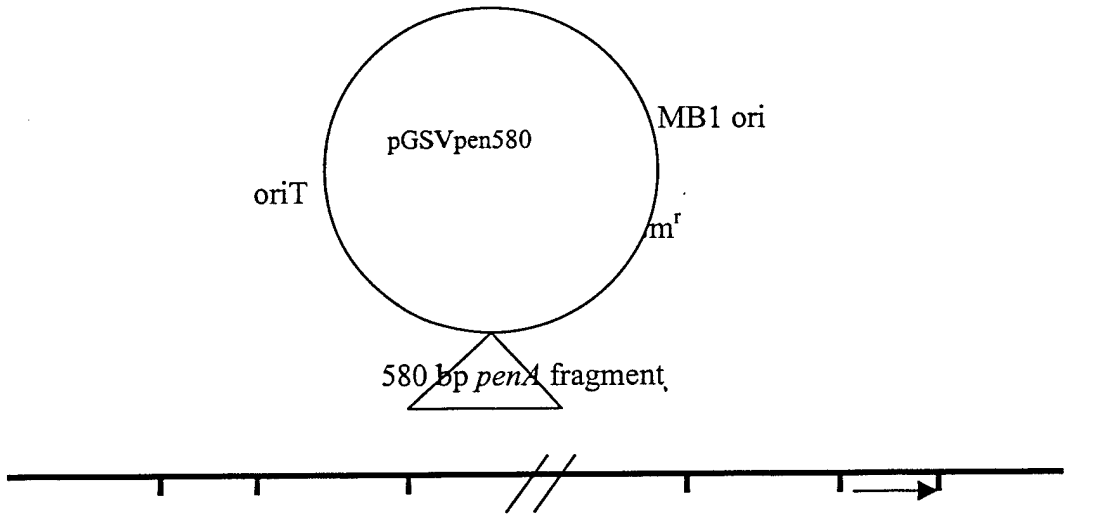


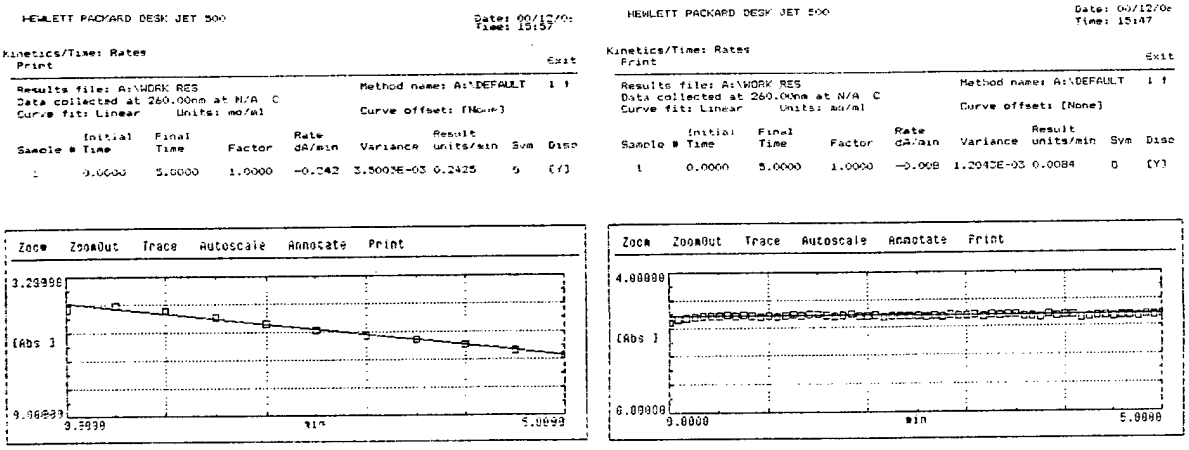
Fig.1 Map shows the pGSVpen580 plasmid and orientation of the inserted vector in the chromosome resulting in the disrupted *penA* gene.

Table 1: Ampicillin and amoxicillin MICs (g/ml) for wild type and mutant *Burkholderia*.

B. thailandensis	Amoxicillin	**B. mallei**	Ampicillin	Amoxicillin
WT E264	>256	WT GB8	>256	>64
Mutant EA-1	6	Mutant GA-1	0.38	0.094
Mutant EA-2	4	Mutant GA-2	0.5	0.094
Mutant EA-3	4			

2. The *penA* gene has been mutated by insertion of a zeocin resistance cassette into the wild type *penA* gene. A *fus*- derivative of this strain will be constructed by insertional inactivation with a ~500 bp internal fragment of the *B. mallei fusE* homolog. The MICs for beta-lactams will be determined in this strain in order to quantify the contribution of each system to beta-lactam resistance.

3. The cloned beta-lactamase gene from *B. pseudomallei* 316a has been expressed in *E. coli* Top10. The beta-lactamase of 316a is identical to one of *B. mallei* GB8. Periplasmic fractions obtained from osmotic disruption demonstrated enzyme activity using nitrocefin and cefazolin as substrates, whereas the periplasmic fraction from wild type Top10 cells did not.



A. B.

Figure 2. The spectrophotometry results showing the cefazolin hydrolysis with TOP 10 (p316a31T) extract (A), but not with wild type TOP 10 extract (B).

Key Research Accomplishments

- We have described a number of genes encoded in pathogenicity island in *B. mallei* and *B. pseudomallei*. We are tremendously excited about the identification of an extracellular polysaccharide component present on these organisms that may very well serve as an ideal vaccine candidate. We are continuing our studies on the purification and characterization of this polysaccharide.

- We have developed a *phoA* mutagenesis procedure that should prove to be tremendously useful for the identification of *B. mallei* mutants deficient in the production of extracellular proteins involved in the virulence of these organisms. Now that we have developed the minitransposon-*phoA* system, it is anticipated that these studies will proceed rapidly.

- We have developed class II MHC knockout mice as an animal model for chronic *B. mallei* infections. These studies should prove to be tremendously important in defining the immunological parameters of glanders as well as providing us an opportunity to test vaccines and chemotherapeutic agents for treating the disease.

- We have shown that the wild type mouse (C57BL/6) can be used as a model for chronic *B. mallei* infection. This strain can also be used in studies such as active immunization, effects of antibiotics, bacterial survival, etc. We do not need MHC knock out mice which need complicated cellular transfer to make them suitable for active immunization study. The mouse model should facilitate vaccine development, because the reagents for mice can be obtained readily.

- We have described a number of parameters important in *B. mallei*-macrophage interactions. Using a genetic approach, we will construct mutants deficient in macrophage uptake and/or survival in an effort to define the parameters associated with *B. mallei*-macrophage interactions.

- We have initiated studies on antibiotic resistance in *B, mallei*. We are in the process of complementing *B. mallei* strains with cloned regions of the *amr* locus from *B. pseudomallei* in order to determine if *B. mallei* lacks certain components of the efflux system.

Reportable Outcomes

- We have published a manuscript describing our studies on the identification of a pathogenicity island in *B. mallei* and *B. pseudomallei*. This manscript is attached as Appendix 1 and has been published in Infection and Immunity.
- We have published a manuscript describing our studies on the development of acid-phosphatase mutants of *Burkholderia spp* and the description of TnPhoA mutagenesis in *Burkholderia spp*. This manuscript is attached as Appendix 2 and has been published in Microbiology.
- We have published a manuscript describing a modified TnPhoA mutagenesis procedure for *Burkholderia spp*. This manuscript is attached as Appendix 3 and has been published in Biotechniques.
- We have collaborated with Dr. David DeShazer on a manuscript describing the details of the genes present in the pathogenicity island in *B. mallei*. This manuscript (DeShazer, D., Waag, D.M., Fritz, D.L., and Woods, D.E. 2001. Identification of a *Burkholderia mallei* Polysaccharide Gene Cluster by Subtractive Hybridization and Demonstration That the Encoded Capsule is an Essential Virulence Determinant) will be published in Microbial Pathogenesis.
- Dr. Alex Bolton and Dr. Chanwit Tribuddharat are postdoctoral fellows currently supported by this contract.
- We successfully obtained a Medical Research Council Operating Grant based on work supported in part by this contract.
- We have developed an animal model of chronic *B. mallei* infection in Class II MHC knockout mice and in C57BL/6 mice.

Conclusions

We have described a method combining PCR-based subtractive hybridization, insertional mutagenesis and an animal infection model for efficiently detecting such virulence genes, and we have applied the method to the pathogens *Burkholderia pseudomallei* and *B. mallei*. The combination of PCR-based subtractive hybridization, insertional inactivation and animal virulence studies should facilitate the identification of relevant virulence determinants from a number of bacterial species; however, we will continue to concentrate on the genes present in the pathogenicity island that we have identified in *B. mallei* and *B. pseudomallei*. In particular, we are tremendously excited about the identification of an extracellular polysaccharide component present on these organisms that may very well serve as an ideal vaccine candidate. We will continue our studies on the purification and characterization of this polysaccharide. We have developed *TnphoA* mutagenesis utilizing pmini-*Tn5phoAMod-OGm*. Preliminary experiments indicate that this system appears more efficient than the pRT733(*TnphoA*), and this system should prove tremendously useful in generating mutants deficient in the production of extracellular proteins important in virulence. Our studies on the use of the class II MHC knockout mice and C57BL/6 mice as an animal model for chronic *B. mallei* infection are tremendously exciting ones. These studies should prove to be significantly important in defining the immunological parameters of glanders as well as providing us an opportunity to test vaccines and chemotherapeutic agents for treating the disease. The overall significance of the work resides in the realization that we are beginning to understand the virulence of *B. mallei*, and we are progressing towards the development of a vaccine that will protect against disease due to this organism.

Appendices

1. Bolton, A.J., and Woods, D.E. 2000. A Self-Cloning Minitransposon *phoA* Gene-Fusion System Promotes the Rapid Genetic Analysis of Secreted Proteins in Gram-Negative Bacteria. Biotechniques. 29(3):472-474.

2. Reckseidler, S.L., DeShazer, D., Sokol, P.A., and Woods, D.E. 2001. Detection of Bacterial Virulence Genes by Subtractive Hybridization: Identification of the Capsular Polysaccharide of *Burkholderia pseudomallei* as a Major Virulence Determinant. Infection and Immunity, In press.

3. Burtnick, M.N., Bolton, A.J., Brett, P.J., Watanabe, D., and Woods, D.E. 2001. Identification of the Acid Phosphatase (*acpA*) Gene Homologues in Pathogenic and Non-pathogenic *Burkholderia spp.* Facilitates Tn*phoA* Mutagenesis. Microbiol. 147:111-120.

INFECTION AND IMMUNITY, Jan. 2001, p. 34–44
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.1.34–44.2001

Vol. 69, No. 1

Detection of Bacterial Virulence Genes by Subtractive Hybridization: Identification of Capsular Polysaccharide of *Burkholderia pseudomallei* as a Major Virulence Determinant

SHAUNA L. RECKSEIDLER,[1] DAVID DeSHAZER,[2] PAMELA A. SOKOL,[1] AND DONALD E. WOODS[1]*

Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Center, Calgary, Alberta, Canada T2N 4N1,[1] and Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702[2]

Received 7 June 2000/Returned for modification 9 August 2000/Accepted 6 October 2000

***Burkholderia pseudomallei*, the etiologic agent of melioidosis, is responsible for a broad spectrum of illnesses in humans and animals particularly in Southeast Asia and northern Australia, where it is endemic. *Burkholderia thailandensis* is a nonpathogenic environmental organism closely related to *B. pseudomallei*. Subtractive hybridization was carried out between these two species to identify genes encoding virulence determinants in *B. pseudomallei*. Screening of the subtraction library revealed A-T-rich DNA sequences unique to *B. pseudomallei*, suggesting they may have been acquired by horizontal transfer. One of the subtraction clones, pDD1015, encoded a protein with homology to a glycosyltransferase from *Pseudomonas aeruginosa*. This gene was insertionally inactivated in wild-type *B. pseudomallei* to create SR1015. It was determined by enzyme-linked immunosorbent assay and immunoelectron microscopy that the inactivated gene was involved in the production of a major surface polysaccharide. The 50% lethal dose (LD_{50}) for wild-type *B. pseudomallei* is <10 CFU; the LD_{50} for SR1015 was determined to be 3.5×10^5 CFU, similar to that of *B. thailandensis* (6.8×10^5 CFU). DNA sequencing of the region flanking the glycosyltransferase gene revealed open reading frames similar to capsular polysaccharide genes in *Haemophilus influenzae*, *Escherichia coli*, and *Neisseria meningitidis*. In addition, DNA from *Burkholderia mallei* and *Burkholderia stabilis* hybridized to a glycosyltransferase fragment probe, and a capsular structure was identified on the surface of *B. stabilis* via immunoelectron microscopy. Thus, the combination of PCR-based subtractive hybridization, insertional inactivation, and animal virulence studies has facilitated the identification of an important virulence determinant in *B. pseudomallei*.**

Burkholderia pseudomallei, the causative agent of melioidosis, is a gram-negative, facultatively anaerobic, motile bacillus that is commonly found in the soil and stagnant waters in Southeast Asia and northern Australia. Infection by *B. pseudomallei* is often due to either direct inoculation into wounds and skin abrasions or to inhalation of contaminated material (11, 24, 30). This would explain the prevalence of the disease among rice farmers as well as helicopter pilots in the Vietnam War who developed melioidosis due to inhalation of contaminated dust (24, 47). Melioidosis may present as an acute pneumonia or an acute septicemia, which is the most severe form of the disease. The disease may also manifest as a chronic infection involving long-lasting suppurative abscesses in numerous sites in the body. Infection with *B. pseudomallei* may even result in a subclinical infection and remain undetected for a number of years. Both the chronic and subclinical forms generally remain undiagnosed until activated by a traumatic event or a decrease in immunocompetence (25).

Both secreted and cell-associated antigens have been identified in *B. pseudomallei*. Cell-associated antigens include exopolysaccharide (EPS) and lipopolysaccharide (LPS) (5, 8, 51). The EPS produced by *B. pseudomallei* is an unbranched polymer of repeating tetrasaccharide units with the structure -3)-2-*O*-acetyl-β-D-Gal*p*-(1-4)-α-D-Gal*p*-(1-3)-β-D-Gal*p*-(1-5)-β-D-KDO*p*-(2- (35, 40). The role of EPS in virulence is unknown, but sera from patients with melioidosis have been shown to contain antibodies against EPS (51). The LPS of *B. pseudomallei* has been reported to contain two types of O-polysaccharide moieties termed type I O-PS and type II O-PS (27, 41). Type II O-PS is an unbranched heteropolymer with repeating D-glucose and L-talose residues with the structure -3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1-, in which approximately 33% of the talose residues contain 2-*O*-methyl and 4-*O*-acetyl substituents, while the other L-talose residues contain only 2-*O*-acetyl substituents. Type II O-PS has been shown to be involved in serum resistance (17). Mutants lacking in type II O-PS were found to be sensitive to the bactericidal activities of 30% normal human serum. Type II O-PS mutants also demonstrated reduced virulence in three animal models of *B. pseudomallei* infection (17). Type I O-PS is an unbranched homopolymer with the structure -3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1-. The role for this polysaccharide in infection was previously undefined.

B. thailandensis is a nonpathogenic soil organism originally isolated in Thailand (6). Based on biochemical, immunological, and genetic data, *B. pseudomallei* and *B. thailandensis* are closely related species. However, these two organisms differ in a number of ways and have been classified into two different species (7). The rRNA sequence of *B. thailandensis* differs from that of *B. pseudomallei* by 15 nucleotides, and there are significant differences in genomic macrorestriction patterns be-

* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Center, 3330 Hospital Dr., NW, Calgary, Alberta, Canada T2N 4N1. Phone: (403) 220-2564. Fax: (403) 283-5241. E-mail: woods@ucalgary.ca.

tween these organisms (10). The biochemical profiles of these two species differ in that *B. thailandensis* can utilize L-arabinose whereas *B. pseudomallei* does not (7, 62). The most distinct difference between these two species, however, is their relative virulence. The 50% lethal dose (LD_{50}) for *B. pseudomallei* in the Syrian hamster model of acute melioidosis is <10 organisms, whereas the LD_{50} for *B. thailandensis* is approximately 10^6 organisms (7). It has also been shown that the two species can be differentiated based on their propensity to cause disease in humans. Environmental strains isolated in Thailand that are able to assimilate L-arabinose are not associated with human infection, whereas clinical isolates are not able to utilize L-arabinose (54).

To identify the genetic determinants that confer enhanced virulence in *B. pseudomallei*, a method combining subtractive hybridization, insertional mutagenesis, and animal virulence studies was developed. The described method should aid in the identification of virulence factors in pathogenic bacteria and provide further insights into microbial diversity and evolution.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *B. pseudomallei*, *B. thailandensis*, *B. cepacia*, and *Escherichia coli* were grown at 37°C on Luria-Bertani (LB) broth base (Becton Dickinson) agar plates or in LB broth. *B. mallei* was grown at 37°C on LB plates or in LB broth supplemented with 4% glycerol and at pH 6.8. For animal studies, *B. pseudomallei* and *B. thailandensis* cultures were grown at 37°C in TSBDC medium (6). When appropriate, antibiotics were added at the following concentrations: 50 μg of tetracycline, 100 μg of streptomycin, 100 μg of polymyxin B, 100 μg of trimethoprim, 25 μg of gentamicin, and 25 μg of kanamycin per ml for *B. pseudomallei* and 100 μg of ampicillin, 25 and 50 μg of kanamycin, 15 μg of tetracycline, and 1.5 mg of trimethoprim per ml for *E. coli*.

Construction and screening of subtractive hybridization libraries. Subtractive hybridization between *B. pseudomallei* and *B. thailandensis* was carried out using a PCR-Select bacterial genome subtraction kit (Clontech) as recommended by the manufacturer except that the hybridization temperature was increased from 63°C to 73°C due to the high G+C content in the genomes of these species. In construction of the subtractive hybridization library, *B. pseudomallei* genomic DNA was used as the tester and *B. thailandensis* genomic DNA was used as the driver. The secondary PCR products obtained were cloned into pZErO-2.1 (Invitrogen) and pPCR (Table 1) and were enriched for *B. pseudomallei*-specific sequences. The subtraction library was screened by sequencing of the tester-specific DNA fragments. The library containing random clones was diluted in sterile phosphate-buffered saline (PBS) to 10^{-6}, and 100 μl was plated on LB plates containing kanamycin (50 μg/ml) and 1 mM isopropylthio-β-D-galactoside (IPTG). Individual colonies were picked and grown overnight at 37°C in LB with kanamycin (50 μg/ml). Plasmid DNA was isolated using a miniprep plasmid isolation kit (Qiagen).

DNA sequencing and analysis. Automated DNA sequencing was performed by ACGT (Northbrook, Ill.) and the University of Calgary Core DNA Services. The M13 forward primer (dGTAAAACGACGGCCAGT) was used to initiate sequence reactions with the subtractive hybridization clones. DNA flanking the Tn*5*-OT182 insertions was sequenced using the previously described primers OT182-LT and OT182-RT (16). The DNA flanking the insertion of pSR1015 was sequenced using the pSKM11 primer (38). DNA and protein sequences were analyzed with DNASIS for IBM and with the ORF Finder program at the National Center for Biotechnology Information (NCBI). DNA sequences were analyzed for homology using the BLASTX program through GenBank at NCBI.

Cloning of a subtractive hybridization product and mobilization into wild-type *B. pseudomallei*. The DNA insert from pDD1015 was cloned as a *Kpn*I-*Xho*I fragment into a mobilizable suicide vector, pSKM11 (Table 2). The 373-bp fragment was ligated to pSKM11 digested with the same enzymes to create pSR1015. SM10(pSR1015) was conjugated with *B. pseudomallei* 1026b using a previously described protocol (16).

Animal studies. The animal model of acute *B. pseudomallei* infection has been previously described (18). Syrian hamsters (females, 6 to 8 weeks) were injected intraperitoneally with 100 μl of one of a number of serial dilutions of logarithmic-phase cultures in sterile PBS. The five control animals were inoculated with 10^1 CFU of wild-type *B. pseudomallei*. The test animals (five per dilution) were inoculated with either 10^1, 10^2, or 10^3 CFU of the mutant strain, SR1015. Blood from two of the test animals was diluted and plated on Ashdown medium with and without the addition of tetracycline (50 μg/ml) to verify the stability of pSR1015 (7). For determination of LD_{50} for SR1015, hamsters (five per group) were inoculated with 10^3, 10^4, 10^5, and 10^6 CFU. After 48 h, the LD_{50} was calculated (42).

Immunoassays. Immunogold electron microscopy was performed as previously described (17). Samples for Western blot analysis were prepared as previously described (8). Immunoassay was performed with a 1:250 dilution of the primary antibody, polyclonal rabbit antiserum raised to a *B. pseudomallei* O-PS–flagellin protein conjugate (5, 8). The secondary antibody used was horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma). Enzyme-linked immunosorbent assays (ELISAs) for the presence of EPS were carried out as previously described, using the EPS-specific monoclonal antibody 3015 at a dilution of 1/100 (17, 51, 52). Tn*5*-OT182 mutants were screened by ELISA according to the same protocol with rabbit polyclonal sera specific for an O-PS–flagellin conjugate containing antibodies to type I O-PS, type II O-PS, and flagella (5).

Southern hybridization. For Southern hybridization, *Sst*I digests of genomic DNA from *B. pseudomallei* 1026b and SR1015, *B. thailandensis* E264, *B. mallei* NCTC 10260, *B. cepacia* CEP509 (genomovar I) and K56-2 (genomovar III), *B. stabilis* LMG14294 and LMG7000, *B. vietamiensis* LMG10929, and *B. multivorans* C5393 were transferred to GeneScreen *Plus* membranes (Du Pont Canada, Lachine, Quebec, Canada), and hybridization was performed at 65°C in 15 ml of 1% sodium dodecyl sulfate (SDS)–10% dextran sulfate–salmon sperm DNA (0.1 mg/ml) according to the manufacturer's recommendations. The 0.4-kb *Kpn*I-*Xho*I fragment from pDD1015 was used as a probe and labeled with [^{32}P]dCTP using an oligonucleotide labeling kit (Pharmacia Biotech, Inc., Baie d'Urfe, Quebec, Canada).

Tn*5*-OT182 mutagenesis and screening for type I O-PS mutants. To screen for mutants deficient in type I O-PS, it was first necessary to create a strain that produced only type I O-PS. This was necessary as the antiserum available was polyclonal antiserum to a flagellin–O-PS conjugate that contains antibodies to flagellin, type I O-PS, and type II O-PS. Therefore, we constructed a strain that was lacking in type II O-PS and flagella, SR1001. Allelic exchange was carried out using two strains that were previously constructed in the laboratory. The donor strain, SM10λ*pir*(pPB611::Gm), has a plasmid containing a copy of the *wbiE* gene, involved in the synthesis of type II O-PS, which has been mutated by the insertion of a gentamicin resistance (Gm) cassette (17). The recipient strain, PB401, is a *B. pseudomallei* strain that has a deletion in the *fliC* gene. SM10λ*pir* (pPB611::Gm) was conjugated to PB401, and transconjugants were selected for by plating on LB containing gentamicin, kanamycin, and polymyxin B. Transconjugants that were Sm^r, Gm^r, and Km^s were selected (to select for loss of the vector, pKAS46), and one was designated SR1001. Transposon mutagenesis of SR1001 was performed with Tn*5*-OT182 according to a previously described protocol (16) except that transconjugants were selected on plates containing gentamicin and tetracycline. Transposon mutants were inoculated into 96-well plates containing 200 μl of LB with gentamicin and tetracycline and grown overnight at 37°C at 250 rpm. A negative growth control well was included for each plate. The wells of a 96-well plate were coated with 10 μl of bacteria and 90 μl of coating buffer (0.05 M carbonate buffer [pH 9.6]), and ELISA was carried out as previously described (5). The primary antibody, polyclonal rabbit antiserum to a *B. pseudomallei* O-PS–flagellin protein conjugate, was added at a dilution of 1:1,000. The secondary antibody, a goat anti-rabbit IgG-peroxidase conjugate (Sigma), was added at a dilution of 1:1,000. The plates were developed with an HRP color development reagent (Bio-Rad Laboratories) for 30 min. The optical density at 405 nm (OD_{405}) was determined using an ELISA reader. *B. pseudomallei* 1026b was included as a positive control, and *E. coli* DH5α was included as a negative control. Transposon mutants that had OD_{405} readings comparable to the negative control (OD_{405} = <0.100) were chosen for further analysis.

Construction of allelic exchange mutants. Allelic exchange was carried out as previously described (17). The allelic exchange vector used in these experiments was pKAS46, an allelic exchange vector based on *rpsL* for counterselection (49). *B. pseudomallei* DD503, a double mutant that contains the Δ*amrR-oprA* and *rpsL* mutations, was the recipient strain used for all allelic exchange experiments (17, 39). All genes in these experiments were mutated by the insertion of a self-cloning trimethoprim resistance (Tp) cassette from p34EoriTp (P. J. Brett, D. DeShazer, and D. E. Woods, unpublished data). For each allelic exchange experiment, SM10λ*pir* transformed with pKAS46 containing the mutated allele was conjugated to *B. pseudomallei* DD503 as described above for the construction of SR1001 except that the transconjugants were plated on polymyxin B,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
SM10	Mobilizing strain; transfer genes of RP4 integrated in chromosome; Km^r Sm^s	48
SM10λ*pir*	SM10 with a λ prophage carrying the gene encoding the π protein	37
SURE	e14$^-$ (*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)*171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC*::Tn*5 uvrC* [F′ *proAB lacI*^{q} ZΔM15 Tn*10*] Kan^r Tet^r	Stratagene
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Bethesda Research Laboratories
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL*(Str^r) *endA1 nupG*	Invitrogen
XL10-Gold	Tet^{r}Δ (*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte[F′ *proAB lacI*^{q} Z ΔM15 Tn*10* (Tet^r) Amy Cam^r]	Stratagene
B. pseudomallei		
1026b	Clinical isolate; Km^r Gm^r Sm^r Pm^r Tc^s Tp^s	16
SR1015	1026b(pSR1015); Sm^r Tc^r	This study
SR1016	1026b(pSR1016); Sm^r Tc^r	This study
DD503	1026b derivative; allelic exchange strain; Δ(*amrR-oprA*)(Km^s Gm^s Sm^s) *rpsL* (Sm^r)	39
PB401	DD503 derivative; Δ*fliC*	Brett et al., unpublished
SR1001	DD503 derivative; Δ*fliC wbiE*::*aacC1* Gm^r	This study
SLR5	SR1001 derivative; *wcbB*::Tn*5*-OT182 Tc^r	This study
SLR8	SR1001 derivative; *wzt2*::Tn*5*-OT182 Tc^r	This study
SLR13	SR1001 derivative; *wcbP*::Tn*5*-OT182 Tc^r	This study
SLR18	SR1001 derivative; *wcbE*::Tn*5*-OT182 Tc^r	This study
SLR19	SR1001 derivative; *wcbH*::Tn*5*-OT182 Tc^r	This study
SR201::Tp	DD503 derivative; *wcbC*::Tp	This study
SR202::Tp	DD503 derivative; *wcbA*::Tp	This study
SR203::Tp	DD503 derivative; *yafJ*::Tp	This study
B. thailandensis E264	Soil isolate; LPS contains only type II O-PS	7
B. mallei NCTC 10260		
B. cepacia complex		
B. cepacia CEP509 (genomovar I)	CF[a] isolate, Australia	33
B. multivorans C5393 (formerly *B. cepacia* genomovar II)	CF isolate, Vancouver, Canada	33
B. cepacia K56-2 (genomovar III)	CF isolate, Toronto, Canada	15
B. stabilis (formerly *B. cepacia* genomovar IV)		
CEP0717	CF isolate, Calgary, Canada	H. Rabin
CEP0467	CF isolate, Edmonton, Canada	E. Mahenthiralingam
J687	Non-CF isolate, France	56
CEP0726	CF isolate, Calgary, Canada	H. Rabin
LMG14291	CF isolate, Belgium	56
LMG7000	Blood isolate, Sweden	56
LMG14294	CF isolate, Belgium	33
B. vietnamiensis (also known as *B. cepacia* genomovar V) LMG10929	Rice root isolate, Vietnam	33
Plasmids		
pSKM11	Positive selection cloning vector; IncP *mob*; ColE1 *ori*; Ap^r Tc^s	38
pPCR	pBluescript II SK(+) derivative; Ap^r	3
pZErO-2.1	Positive selection cloning vector; ColE1; Km^r	Invitrogen
pPCR2.1-TOPO	Topoisomerase-mediated cloning vector; Ap^r Km^r	Invitrogen
pDD1015	Subtractive hybridization product cloned into pZErO-2.1; Km^r	This study
pDD1016	Subtractive hybridization product cloned into pZErO-2.1; Km^r	This study
pSR1015	*Kpn*I-*Xho*I fragment from pDD1015 cloned into pSKM11 Ap^r Tc^r	This study
pSR1016	*Kpn*I-*Xho*I fragment from pDD1016 cloned into pSKM11; Ap^r Tc^r	This study
pSR1015Bg	8-kb *Bgl*II fragment from SR1015 obtained by self-cloning; Ap^r Tc^r	This study
pOT182	pSUP102(Gm)::Tn*5*-OT182; Cm^r Gm^r Ap^r Tc^r	36
pSLR5B	8-kb *Bam*HI fragment from SLR5 obtained by self-cloning; Ap^r	This study
pSLR5H	10-kb *Hin*dIII fragment from SLR5 obtained by self-cloning; Ap^r	This study
pSLR13H	9-kb *Hin*dIII fragment from SLR13 obtained by self-cloning; Ap^r	This study
p34E-oriTp	Vector containing self-cloning Tp cassette; *dhfrllb*-p15A *oriV*	Brett et al., unpublished

[a] CF, cystic fibrosis.

TABLE 2. Recombinant plasmids in the *B. pseudomallei-B. thailandensis* subtraction library

Plasmid	Vector	Insert size (bp)	% G+C	Homologue[a]
pDD1000	pPCR	326	51	DprA
pDD1001	pPCR	800	44	None
pDD1002	pPCR	434	50	GuaA
pDD1003	pPCR	346	51	None
pDD1004	pPCR	800	44	None
pDD1005	pPCR	531	46	Mob protein
pDD1006	pPCR	353	48	None
pDD1007	pZErO-2.1	325	51	None
pDD1008	pZErO-2.1	250	44	None
pDD1009	pZErO-2.1	350	52	None
pDD1012	pZErO-2.1	505	47	None
pDD1015	pZErO-2.1	373	52	WbpX
pDD1016	pZErO-2.1	259	46	None
pDD1017	pZErO-2.1	100	50	None
pDD1018	pZErO-2.1	433	50	None

[a] Homologues and references: *Haemophilus influenzae* DprA, Karudapuram et al. (26); *Bacillus subtilis* GuaA, Mantsala and Zalkin (34); *Bordetella bronchiseptica* plasmid pBBR1 mobilization (Mob) protein, Antoine and Locht (1); *Pseudomonas aeruginosa* WbpX, Rocchetta et al. (45).

kanamycin, and trimethoprim. The Pm^r Km^r Tp^r transconjugants were subsequently transferred to plates containing streptomycin to select for the loss of pKAS46. Mutant alleles were confirmed by self-cloning and sequencing.

DNA manipulation. Restriction enzymes and T4 DNA ligase were purchased from Life Technologies (Burlington, Ontario, Canada) and New England Biolabs (Mississauga, Ontario, Canada) and used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified using a GeneClean II kit (Bio 101, Vista, Calif.) or Qiagen (Mississauga, Ontario, Canada) gel extraction kit. Chromosomal DNA was isolated using a previously described protocol (60). The self-cloning of *B. pseudomallei* flanking DNA from Tn*5*-OT182 mutants and from SR1015 was performed as described previously (16).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in the GenBank database under accession number AF228583.

RESULTS

Construction and screening of the *B. pseudomallei* subtraction library. Subtractive hybridization was carried out between the virulent *B. pseudomallei* and the weakly virulent *B. thailandensis* in order to isolate DNA sequences encoding for virulence determinants unique to *B. pseudomallei*. The genomic DNA sample from *B. pseudomallei* containing the sequences of interest was known as the tester DNA, and genomic DNA from *B. thailandensis*, the reference sample, was called the driver DNA. Tester and driver DNAs were digested and subjected to two rounds of hybridization. The remaining unhybridized sequences were considered tester-specific sequences. To enrich for tester-specific sequences, excess driver DNA was added in the hybridizations. The tester-specific sequences were then amplified by PCR and cloned into pPCR or pZErO-2.1 (Table 1).

Screening of the subtraction library revealed a number of DNA sequences unique to *B. pseudomallei*. Fifteen distinct plasmid inserts from the library were sequenced (Table 2). The DNA inserts ranged from 100 to 800 bp in length and were found to contain an average G+C content of approximately 44 to 52%, which is considerably lower than the 68% G+C content of the *B. pseudomallei* chromosome. The DNA sequences were analyzed using the NCBI BLASTX program, and only four of the sequences had homology to predicted proteins present in the GenBank database. One of the plasmid inserts, pDD1000, had homology to DprA, a protein required for chromosomal DNA transformation in *Haemophilus influenzae* (26). Another insert, pDD1005, had homology to a mobilization protein found in small plasmids (1). The third, pDD1015, was found to share limited homology with WbpX, a glycosyltransferase, from *Pseudomonas aeruginosa* (45). The fourth, pDD1002, demonstrated homology to GuaA, a GMP synthetase, from *Bacillus subtilis* (34).

Insertional inactivation of the glycosyltransferase gene in wild-type *B. pseudomallei*. The 373-bp DNA insert from pDD1015 was cloned into a mobilizable suicide vector, pSKM11 (Table 1). The resulting plasmid, pSR1015, was mobilized into wild-type *B. pseudomallei* 1026b to create the mutant strain SR1015. Since the insert from pDD1015 was found to demonstrate homology to a glycosyltransferase from *P. aeruginosa*, it was postulated that it might encode a protein involved in carbohydrate synthesis.

To define the phenotype of SR1015, an ELISA was performed with the EPS-specific monoclonal antibody 3015, and *B. pseudomallei* 1026b and SR1015 were both found to contain EPS (data not shown) (52). SR1015 was also shown to contain type II O-PS and to be serum resistant (data not shown). Immunogold electron microscopy studies using rabbit polyclonal sera specific for a type I O-PS–flagellin conjugate was performed on the parent strain, 1026b, and SR1015 (Fig. 1). *B. pseudomallei* 1026b reacted with antibodies to both flagellin and type I O-PS, as was evident by the distribution of gold particles around the bacterial surface and extending out along the flagella (Fig. 1A). The distribution of the gold particles around the outer surface of the bacteria corresponds to the type I O-PS structure, which is known to extend out beyond the type II O-PS. Unlike *B. pseudomallei* 1026b, SR1015 reacted only with the antibodies to flagellin, as the gold particles were found associated only with the flagella (Fig. 1B). *B. thailandensis*, the negative control, did not react with the antibodies either to flagellin or to type I O-PS (Fig. 1C). Western blot analysis of proteinase K-digested whole cells from *B. pseudomallei* 1026b, *B. thailandensis* E264, and *B. pseudomallei* SR1015 using rabbit polyclonal sera raised to O-PS–flagellin protein conjugate confirmed the lack of type I O-PS in SR1015 (Fig. 2). Type I and type II O-PS were stained in *B. pseudomallei* 1026b, while only type II O-PS was stained in the lanes corresponding to *B. pseudomallei* SR1015 and *B. thailandensis*. These results indicated that we had identified and insertionally inactivated a gene involved in the synthesis of the type I O-PS of *B. pseudomallei*.

SR1015 is avirulent in the animal model of infection. SR1015 was tested for virulence in the Syrian hamster model of acute septicemic melioidosis. The LD_{50} for SR1015 after 48 h was 3.5×10^5 CFU, while the LD_{50} of the parent strain, 1026b, was <10 CFU. The LD_{50} for SR1015 was similar to that for the weakly virulent *B. thailandensis* (6.8×10^5 CFU). This demonstrates that SR1015 is severely attenuated for virulence in this animal model of melioidosis and that type I O-PS is a major virulence determinant of *B. pseudomallei*.

Cloning and sequencing of the genetic loci required for type I O-PS production and export. Two methods were used to clone the genes involved in the production and export of type I O-PS. The DNA flanking the insertion of pSR1015 was cloned from SR1015 and sequenced. We also used transposon

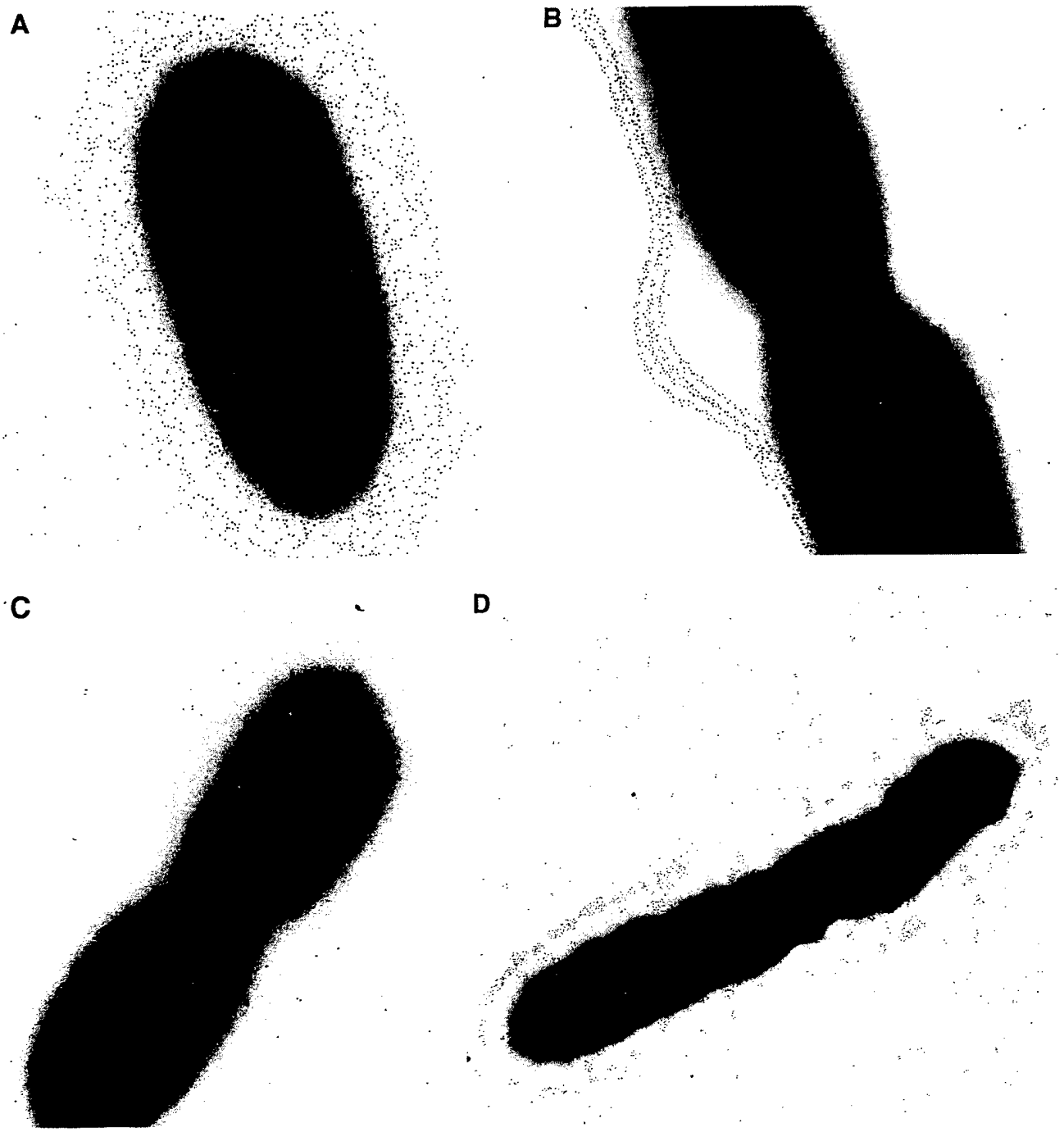


FIG. 1. Immunogold electron microscopy of *B. pseudomallei* 1026b (A) and SR1015 (B), *B. thailandensis* E264 (C), and *B. stabilis* LMG7000 (D). Bacteria were reacted with polyclonal rabbit antiserum directed against an O-PS–flagellin protein conjugate absorbed with *B. thailandensis* E264 to remove the antibodies directed against type II O-PS, washed, and reacted with a goat anti-rabbit IgG-gold (5 nm) conjugate. Original magnification, ×30,000.

mutagenesis to clone the genes involved in production of the polysaccharide; this was done to obtain any unlinked genes that may be involved in polysaccharide production. Approximately 1,300 transposon mutants were screened for loss of type I O-PS by ELISA. Six mutants were identified, and the DNA flanking the transposon insertion was cloned and sequenced. The Tn*5*-OT182 mutants SLR5, SLR8, SLR13, SLR18, and SLR19 mapped to the same region of the chromosome (Fig. 3). Sequence analysis of the cloned fragments revealed the presence of 20 potential open reading frames involved in the synthesis and export of type I O-PS (Fig. 3). The open reading frames that predicted proteins involved in polysaccharide bio-

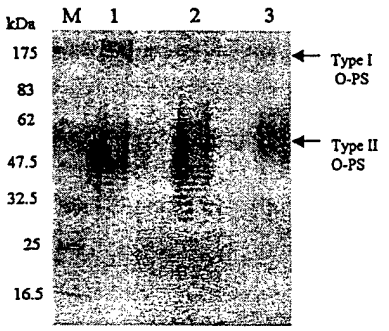


FIG. 2. Western blot analysis of LPS isolated from *B. pseudomallei* 1026b and SR1015 and *B. thailandensis* E264. Bacteria were reacted with proteinase K, subjected to SDS-polyacrylamide gel electrophoresis, electroblotted, and reacted with polyclonal rabbit antiserum raised to an O-PS–flagellin protein conjugate from *B. pseudomallei*. Lane M, prestained protein molecular weight standards (New England Biolabs); lane 1, *B. pseudomallei* 1026b; lane 2, *B. pseudomallei* SR1015; lane 3, *B. thailandensis* E264. The apparent molecular masses of the prestained proteins are indicated.

synthesis were found to demonstrate homology to proteins involved in the synthesis of a polysaccharide structure composed primarily of mannose (Table 3). The other reading frames in the locus predicted proteins involved in the transport of capsular polysaccharides in a variety of bacteria, particularly those that produce group 2 and group 3 capsular polysaccharides (Table 3 and reference 59). The genes responsible for the production of type I O-PS was found to be similar to other loci encoding for capsular polysaccharides in that they are divergently transcribed (Fig. 3 and reference 44). The gene cluster involved in the production of this polysaccharide is also similar to group 3 capsule gene clusters in that there are no genes encoding KpsF and KpsU, which are present in group 2 capsule gene clusters (59). However, the organization of the *B. pseudomallei* type I O-PS gene cluster differs in that it does not contain two export regions flanking a single biosynthetic region as seen in other group 3 capsule polysaccharide clusters (12). The biosynthetic genes identified thus far are not organized into one continuous transcriptional unit; instead, *wcbB*, *manC*, and *wcbP* are separated from the rest of the biosynthetic genes. Another interesting feature is that *kpsC* is usually found next to *kpsS* in other group 2 and 3 clusters, unlike the case for *wcbA* and *wcbO* in *B. pseudomallei* (Fig. 3 and reference 59). The promoter sequences of the transcriptional regions of the type I O-PS cluster have yet to be identified. The overall G+C content of this region is about 58%, lower than the G+C content of the rest of the chromosome (68%). The low G+C content in these clusters suggests that polysaccharide genes have a common origin and may have been transferred horizontally between species (21).

The genes involved in the production of the type I O-PS have been named according to the bacterial polysaccharide gene nomenclature scheme (43). The gene products associated with the type I O-PS cluster and their homologues are listed in Table 3. Mutations constructed in a number of these genes have confirmed their role in the production of type I O-PS. One gene that is required for the production of the polysaccharide is *wcbA* (Fig. 3; Table 3). The *wcbA* gene and *wcbO* predict proteins that demonstrate homology to the KpsC and KpsS proteins of *E. coli* and the LipA and LipB proteins of *Neisseria meningitidis*, respectively (Table 3). These proteins are involved in the processing and export of capsular polysaccharide in these organisms (22, 44). To confirm the role of the *wcbA* gene in capsule production, an allelic exchange mutant was constructed by the insertion of a Tp cassette (Fig. 3). The resulting strain, SR202::Tp, did not produce polysaccharide and demonstrated attenuated virulence in the hamster model, similar to SR1015 (data not shown).

The *wcbC*, *wcbD*, *wzm2*, and *wzt2* genes encode proteins that demonstrate homology to proteins involved in the transport of capsular polysaccharides (Table 3) (20, 28, 46). The *wcbC* gene predicts a protein that shares homology with KpsD, a periplasmic protein involved in capsule polysaccharide export in *E. coli* (21, 28, 61). An isogenic mutant was constructed by the insertion of a Tp cassette into the *wcbC* gene (Fig. 3). The resulting strain, SR201::Tp, was still virulent in the hamster model, and type I O-PS was detected on Western blots (data not shown). This is in contrast to the phenotype observed with *E. coli kpsD* mutants (61). The gene products encoded by *wzm2* and *wzt2* are homologous to the KpsM and KpsT proteins of *E. coli*, CtrC and CtrD of *N. meningitidis*, and BexA and BexB of *H.*

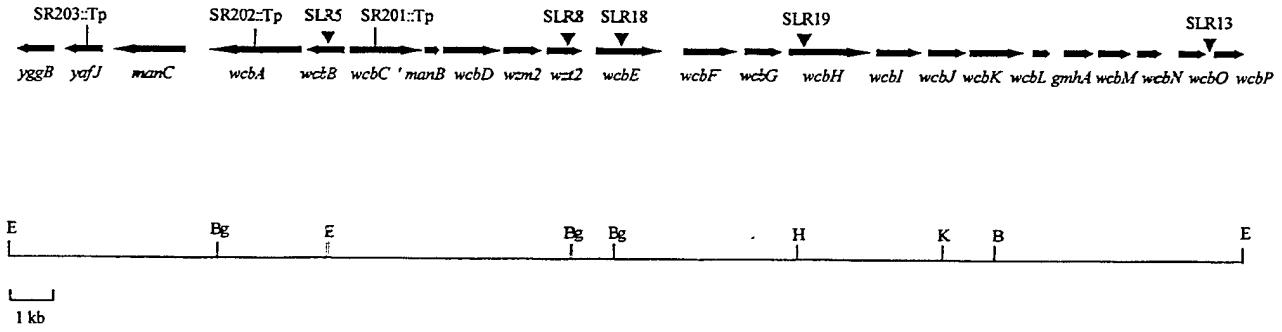


FIG. 3. Organization of the chromosomal region containing the genes responsible for the synthesis and export of type I O-PS in *B. pseudomallei*. The upper part shows the locations of the genes. The direction of transcription is represented by arrows, and gene names are indicated. The locations of Tn*5*-OT182 insertions are represented by triangles. Mutants constructed by allelic exchange are shown. The straight line indicates insertion of the Tp cassette into the gene of interest. The horizontal line below the genetic map represents *B. pseudomallei* chromosomal DNA; the locations of relevant restriction endonuclease recognition sites (Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; B, *Bam*HI) are shown.

TABLE 3. Genes involved in the production and export of type I O-PS in *B. pseudomallei* and homologous proteins located in the nonredundant sequence database

Gene	Size (bp)	Homologue, strain	Putative function	% Identity	% Similarity
manC	1,427	ManC, *Escherichia coli*	Mannose-1-phosphate guanyltransferase	55	70
		ManC, *Salmonella enterica* serovar Typhimurium		54	68
		ManC, *Klebsiella pneumoniae*		48	65
wcbA	2,015	KpsC, *E. coli*	Capsule polysaccharide export protein	37	52
		LipA, *Neisseria meningitidis*		39	55
		PhyA, *Pseudomonas multocida*		35	51
wcbB	1,097	WbpX, *P. aeruginosa*	Glycosyltransferase/mannosyltransferase	33	49
		ManB, *Aquifex aeolicus*		26	44
		MtfA, *Archaeoglobus fulgidis*		30	47
wcbC	1,163	KpsD, *E. coli*	Capsule export periplasmic protein	26	39
		CtrA, *N. meningitidis*		40	58
		BexD, *Haemophilus influenzae*		37	58
'manB	203	XanA, *Xanthomonas campestris*	Phosphomannomutase	35	48
		ManB, *K. pneumoniae*		29	49
		Rfk9, *E. coli*		35	53
wcbD	1,148	BexC, *H. influenzae*	Capsule export inner membrane protein	40	62
		CtrB, *N. meningitidis*		38	60
		KpsE, *E. coli*		26	48
wzm2	410	CtrC, *N. meningitidis*	Capsule export inner membrane protein	53	73
		BexB, *H. influenzae*		28	50
		KpsM, *E. coli*		28	50
wzt2	746	BexA, *H. influenzae*	ATP-binding protein	59	75
		CtrD, *N. meningitidis*		57	72
		KpsT, *E. coli*		46	66
wcbE	1,523	MtfB, *A. aeolicus*	Mannosyltransferase/glycosyltransferase	28	42
		WbpX, *P. aeruginosa*		38	55
		ManB, *Synechocystis* spp.		30	48
wcbF	1,379	Putative, *Homo sapiens*	Heparan-sulfate 6-sulfotransferase	24	39
		Putative *Arabidopsis thaliana*	En/Spm transposon protein	37	52
wcbG	941	SyfB, *Helicobacter pylori*	Phenylalanyl-tRNA synthetase	29	46
wcbH	1,796	MtfA, *Archaeoglobus* spp.	Mannosyltransferase/glycosyltransferase	23	40
		Putative, *Synechocystis* spp.		25	42
		Putative, *Streptomyces coelicolor*		28	41
wcbI	1,187	NifQ, *Enterobacter agglomerans*	Nitrogen fixation protein	28	41
wcbJ	842	Rbd1, *Methanobacterium thermoautotrophicum*	dTDP-4-dehydrorhamnose reductase	23	39
		RmlD, *Mycobacterium tuberculosis*		24	40
wcbK	1,013	Gm4D, *E. coli*	GDP-mannose dehydratase	29	48
		Gm4D, *Yersinia pseudotuberculosis*		32	49
		Gm4D, *Vibrio cholerae*		30	48
wcbL	1,040	Putative, *Campylobacter jejuni*	Sugar kinase	40	56
		Rv0115, *M. tuberculosis*	Lincomycin production	39	52
		LmbP, *Synechocystis* spp.		24	36
gmhA	593	LpcA, *H. pylori*	Phosphoheptose isomerase	50	68
		GmhA, *Methanococcus jannaschii*		54	72
		LpcA, *H. influenzae*		45	60
wcbM	692	RmlA2, *M. tuberculosis*	Mannose-1-phosphate guanyltransferase	32	46
		Putative, *C. jejuni*	Sugar-phosphate nucleotidyltransferase	41	58
wcbN	353	YaeD, *E. coli*	Hypothetical intergenic protein	40	62
		YaeD, *H. influenzae*	Hypothetical intergenic protein	40	58
wcbO	731	KpsS, *E. coli*	Capsule polysaccharide export protein	34	46
		PhyB, *Pasteurella multocida*		29	45
		LipB, *N. meningitidis*		27	44
wcbP	1,950	YooP, *M. tuberculosis*	Oxidoreductase	37	52
		HetN, *Anabaena* spp.		34	52

influenzae (Table 3). These proteins are ATP-binding cassette (ABC) transporters that comprise an inner membrane polysaccharide export system (50). The *wzm2* and *wzt2* gene products of *B. pseudomallei* likely comprise an ABC transporter system that is involved in the transport of the type I O-polysaccharide across the cytoplasmic membrane. The termination codon of the *wzm2* gene overlaps the initiation codon of the *wzt2* gene, suggesting that these two genes are translationally coupled. The *kpsM* and *kpsT* genes of *E. coli* are organized into a single transcriptional unit, and both genes are translationally coupled (44). These genes have been designated *wzm2* and *wzt2* since *wzm* and *wzt* have previously been identified and are associated with the type II O-PS gene cluster (17). A hydrophobicity plot of the predicted *wzm2* gene product revealed a hydrophobic protein with multiple-membrane spanning domains, like KpsM, that may act as an integral membrane protein for the export of polysaccharide (29). Analysis of the primary amino acid sequence of the predicted Wzt2 protein from *B. pseudomallei* has shown that this protein contains a conserved ATP-binding motif, including an A site (GGNGAG KST) and a B site (DCFLIDE) (57). The *wzt2* gene was found to be necessary for the production of type I O-PS in *B. pseudomallei*. In SLR18, the insertion of Tn*5*-OT182 in the *wzt2* gene resulted in a loss of type I O-PS.

The *wcbB*, *wcbE*, and *wcbH* genes encode for proteins that demonstrate homology to different mannosyltranferases or glycosyltransferases from a variety of bacterial species (Table 3). Since type I O-PS is a homopolymer of mannoheptopyranosyl residues, it is likely that these genes are involved in the biosynthesis of this polysaccharide. The *wcbB* gene encodes for a protein with homology to a glycosyltransferase, WbpX, from *P. aeruginosa* as well as to mannosyltransferases from a variety of bacteria (45). The function of glycosyltransferases is to catalyze the sequential transfer of sugar residues from nucleotide precursors to the membrane-bound acceptor, undecaprenol phosphate-P-Glc*p*NAc (58). The *wcbB* gene product is likely involved in the transfer of mannose residues in the synthesis of type I O-PS. This gene was determined to be required for the synthesis of type I O-PS based on two lines of evidence: the insertional inactivation of this gene using pSKM11 rendered the mutant strain, SR1015, negative for type I O-PS production; and a transposon mutant, SLR5 (Fig. 3), lacked type I O-PS due to the insertion of Tn*5*-OT182 in the *wcbB* gene. The *wcbE* and *wcbH* genes both predict proteins with homology to mannosyltransferases and are both required for the production of type I O-PS. This is supported by the fact that the insertion of Tn*5*-OT182 in both the *wcbH* and *wcbE* genes (SLR19 and SLR18, respectively) resulted in mutant strains lacking type I O-PS (Fig. 3). Furthermore, an internal fragment of the *wcbE* gene was cloned into pSKM11 and used to insertionally inactivate this gene in *B. pseudomallei*. The resulting strain, SR1016, was found to lack type I O-PS and demonstrated attenuated virulence in the animal model (data not shown).

Another gene required for the production of type I O-PS is *wcbP*. This gene predicts a protein that shares homology to the YooP protein of *Mycobacterium tuberculosis* (Table 3). The YooP protein has been characterized as a putative oxidoreductase based on sequence comparisons (13). The function of the predicted *wcbP* gene product in *B. pseudomallei* is unclear; however, the insertion of Tn*5*-OT182 into this gene in the mutant strain SLR13 rendered the organism negative for the production of type I O-PS.

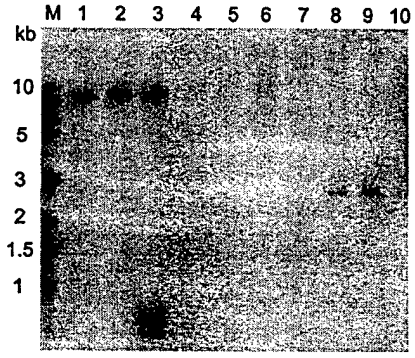


FIG. 4. Southern hybridization analysis of genomic DNA from *Burkholderia* spp. digested with *Sst*I. A 0.4-kb *Kpn*I-*Xho*I fragment from pDD1015 was used as a probe. Lane 1, *B. mallei* NCTC 10260; lane 2, *B. pseudomallei* 1026b; lane 3, *B. pseudomallei* SR1015; lane 4, *B. thailandensis* E264; lane 5, *B. vietnamiensis* LMG10929; lane 6, *B. cepacia* CEP509 (genomovar I); lane 7, *B. cepacia* K56-2 (genomovar III); lane 8, *B. stabilis* LMG14294; lane 9, *B. stabilis* LMG7000; lane 10, *B. multivorans* C5393.

The *yafJ* gene encodes a protein of 278 amino acids that demonstrates homology to the YafJ protein from *E. coli* (Table 3). The YafJ protein is a putative amidotransferase in these organisms (2). The *yggB* gene encodes a protein of 235 amino acids with homology to the YggB protein of *E. coli* (Table 3). The function of this protein is unclear, but it has been defined as a hypothetical 30.9-kDa protein in an intergenic region (2). The G+C contents of these genes are 65.7% for *yafJ* and 65.4% for *yggB*, which is higher than the rest of the polysaccharide cluster and consistent with the G+C content of the *B. pseudomallei* chromosome. Southern blot analysis using these genes as probes has demonstrated their presence in *B. thailandensis* (data not shown); therefore, it is unlikely that these genes are required for the production of type I O-PS (Fig. 3). An allelic exchange mutant containing a Tp cassette in the *yafJ* gene was constructed. The resulting strain, SR203::Tp, was found to be virulent in hamsters (data not shown).

The type I O-PS is also present in *B. mallei* and the *B. cepacia* complex but not in *B. thailandensis*. Southern blot analysis using a probe containing the A-T-rich glycosyltransferase fragment from pDD1015 confirmed that the fragment was present in *B. pseudomallei* 1026b and SR1015 but not in *B. thailandensis* (Fig. 4). *B. mallei* and the *B. cepacia* complex were also tested for the presence of this fragment. It was found that the probe hybridized to an *Sst*I fragment in *B. mallei* and the *B. stabilis* (formerly genomovar IV) strains LMG7000 and LMG14294. The *B. cepacia* complex has recently been divided into two genomovars (*B. cepacia* genomovar I and genomovar III) and three species (*B. multivorans*, *B. vietnamiensis*, and *B. stabilis*) (55, 56). None of the strains tested from *B. cepacia* genomovars I and III, *B. multivorans*, or *B. vietnamiensis* were found to contain this DNA fragment. Southern blot analysis was carried out on five other *B. stabilis* strains: CEP0717, CEP0467, J687, CEP0726, and LMG14291. All of the *B. stabilis* strains tested hybridized to the probe from pDD1015 (data not shown). The presence of type I O-PS was confirmed

in the *B. stabilis* strain LMG7000 by immunoelectron microscopy. As seen in Fig. 1D, *B. stabilis* LMG7000 showed reactivity to the type I O-PS antibodies, but lacked a uniform distribution of the polysaccharide on the cell surface, and therefore appears to produce less of this polysaccharide than *B. pseudomallei* 1026b.

Further Southern blot experiments were carried out to confirm the absence of the type I O-polysaccharide in *B. thailandensis*. *B. thailandensis* was hybridized with probes corresponding to a number of genes involved in the synthesis of the type I O-PS. The following genes were demonstrated by Southern hybridization to be present in *B. pseudomallei* but absent in *B. thailandensis*: *wcbA*, *wcbC*, *wcbD*, *wzm2*, *wzt2*, *wcbE*, *wcbF*, *wcbH*, *wcbK*, *gmhA*, and *wcbO* (data not shown).

DISCUSSION

Although melioidosis is less common outside of Southeast Asia and northern Australia, it may be underdiagnosed in other regions, and it poses a concern due to increased travel and military involvement in regions where the disease is endemic (14). Recently, our attention has been focused on the identification of genetic determinants that contribute to the pathogenesis of *B. pseudomallei* infections. To obtain virulence determinants unique to *B. pseudomallei*, we used subtractive hybridization between this organism and a related nonpathogenic organism, *B. thailandensis*.

Analysis of the subtractive hybridization library revealed that *B. pseudomallei* contains a number of DNA sequences that are not found in *B. thailandensis* (Table 2). One of the subtraction clones, pDD1015, demonstrated weak homology to a glycosyltransferase, WbpX, from *P. aeruginosa* (45). The insert from pDD1015 was cloned into a mobilizable suicide vector, pSKM11, for insertional inactivation of the glycosyltransferase gene in wild-type *B. pseudomallei*. The resulting strain, SR1015, was markedly less virulent than the parent strain in an animal model. This demonstrated that *B. pseudomallei* contains DNA sequences encoding for virulence determinants that are not found in *B. thailandensis* and that the glycosyltransferase gene may encode an important virulence determinant in *B. pseudomallei*. Using antibodies to type I O-PS, we determined that SR1015 harbored a mutation in a glycosyltransferase gene involved in the production of type I O-PS.

Sequence analysis of the DNA flanking the glycosyltransferase gene revealed the presence of at least 20 open reading frames involved in the synthesis and export of type I O-PS (Fig. 3; Table 3). The genes identified encode for proteins that are similar to proteins involved in the biosynthesis and export of capsular polysaccharides, particularly those involved in the production of group 3 capsular polysaccharides. Group 3 capsules include the *E. coli* K10 capsule and may also include the *H. influenzae* group b capsule and the capsule produced by *N. meningitidis* serogroup B (59). Group 3 capsules are always coexpressed with O serogroups, are not thermoregulated, are transported by an ABC-2 exporter system, and do not contain the *kpsU* and *kpsF* genes, and usually the gene clusters map near the *serA* locus (59). Thus far, no *serA* locus that is associated with the type I O-PS cluster has been identified, but this polysaccharide is coexpressed with O antigen and lacks the *kpsU* and *kpsF* genes, and genes encoding for a putative ABC-2 transporter have been identified. The genes involved in the production of group 3 capsules are organized into regions and are divergently transcribed. Regions 1 and 3 are generally conserved and contain genes involved in export of the polysaccharide. These regions flank region 2, which contains the biosynthetic genes and is not conserved between serotypes (44). The genetic organization of the type I O-PS is also similar to that of other capsule gene clusters in that the genes are organized into more than one transcriptional unit and appear to be divergently transcribed. However, the organization of the *B. pseudomallei* type I O-PS cluster differs in that the biosynthetic genes identified thus far are not organized into one biosynthetic region. *yafJ* and *yggB* are likely not involved in the production of type I O-PS since they have a high G+C content (62 to 65%), they are present in *B. thailandensis*, and a mutation in *yafJ* (SR203::Tp) did not reduce virulence in hamsters (data not shown). We are also currently constructing a mutant in the polyketide synthase gene that lies downstream of the *wcbP* gene in order to define this end of the type I O-PS cluster.

The polysaccharide with the structure -3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1- was originally isolated and characterized as an O-PS component of LPS in *B. pseudomallei* and was designated type I O-PS (41). However, our results suggest that this polysaccharide is a capsule rather than an O-PS moiety. The genes involved in the production of this capsule demonstrate strong homology to the genes involved in the production of capsular polysaccharides in many organisms, including *N. meningitidis*, *H. influenzae*, and *E. coli*. In addition, the export genes associated with this cluster are not associated with the previously characterized O-PS gene cluster (17). Western blot analysis of proteinase K cell extracts (Fig. 2) and silver staining (data not shown) have shown that this polysaccharide has a high molecular mass (200 kDa) and lacks the banding pattern seen with O-PS moieties. Studies by our laboratory have indicated that mutants in the production of the core oligosaccharide of the LPS are still capable of producing this polysaccharide (9). Based on the above criteria and the genetic similarity to group 3 capsules, we propose that this polysaccharide is a group 3 capsule.

Capsule production has been correlated with virulence in many bacteria, particularly those causing serious invasive infections of humans (4). Our studies have demonstrated that this capsule is critical for the virulence of *B. pseudomallei*. However, its specific role in infection has yet to be elucidated. A number of functions have been suggested for polysaccharide capsules: prevention of desiccation for transmission and survival, adherence for colonization, resistance to complement-mediated phagocytosis and complement-mediated killing, and resistance to specific host immunity due to a poor antibody response to the capsule (44). Preliminary studies have shown that type I O-PS is not involved in serum resistance. SR1015 was tested for resistance to killing by 30% normal human serum and was found to be resistant to killing (data not shown). Studies to define the role of the capsule in infection are under way.

Genomic DNAs from *B. mallei* NCTC 10260 and seven strains of *B. stabilis* were shown to hybridize to the glycosyltransferase probe from pDD1015. Immunoelectron microscopic analysis demonstrated that *B. stabilis* LMG7000 con-

tained this capsule (Fig. 1). Interestingly, *B. stabilis* LMG7000 was noted to produce less of this polysaccharide than *B. pseudomallei* 1026b and lacked a uniform distribution of the polysaccharide on the cell surface. The importance of the capsule in infection by *B. stabilis* has yet to be elucidated. The results of our study demonstrating the presence of this capsule in *B. stabilis* corresponds with its recent classification as a novel species (56). This capsule may be an additional tool to aid in the identification of *B. stabilis* strains.

Virulence genes of a number of pathogenic bacteria are located on pathogenicity islands (PAIs), regions on the bacterial chromosome that are present in the genome of pathogenic strains but rarely present in those of nonpathogenic strains. The PAIs may range in size from about 30 kb to 200 kb and often differ in G+C content from the remaining bacterial genome; the PAIs are often associated with the carriage of many virulence genes. These genetic units are often flanked by direct repeats and may be associated with tRNA genes or insertion sequence (IS) elements at their boundaries. They may also be associated with the presence of mobility genes, such as IS elements, integrases, transposases, and origins of plasmid replication. These DNA regions are considered to be unstable in that they may be subject to deletion with high frequency or undergo duplications and amplifications (23). A number of PAIs have been described for both gram-positive and gram-negative bacteria, and the application of subtraction hybridization has been used to successfully identify such genetic elements (23, 32). The subtractive hybridization that was carried out between *B. pseudomallei* and *B. thailandensis* led to the identification of a number of sequences that were found to be A-T rich compared to the rest of the *B. pseudomallei* chromosome. This, combined with the fact that insertional mutagenesis of the glycosyltransferase gene identified by this method resulted in an avirulent strain, suggests that we may have identified DNA sequences from a putative PAI and that the capsular polysaccharide gene cluster may be located on this island. It is possible that *B. pseudomallei*, *B. mallei*, and *B. stabilis* acquired DNA encoding for capsule as well as other potential, yet unidentified virulence factors by horizontal transfer recently in evolution. *B. pseudomallei* is known to contain IS elements that are present in *B. cepacia* but not in *B. thailandensis* (31). However, IS elements have not yet been identified in association with the capsule gene cluster. Further studies are under way to determine whether a PAI exists in these organisms and whether the capsule gene cluster is located on such a genetic element.

The identification of bacterial virulence genes has traditionally relied on empirical predictions of putative virulence determinants and inactivation of the genes encoding for these putative virulence determinants by any number of methods, followed by comparisons of virulence between mutant and wild-type infection models (19). Tools such as in vivo expression technology and differential fluorescence technology have been developed to facilitate the identification of expressed sequences under a given set of circumstances within a test host; however, these approaches do not necessarily lead to the identification of virulence determinants (53). The method for identification of virulence genes described herein should be applicable to a broad range of pathogenic bacteria. The combination of PCR-based subtractive hybridization, insertional mutagenesis, and an animal infection model provides for the efficient detection of virulence genes. While we have applied the method to the pathogen *B. pseudomallei* in our current studies, it could be applied to any species and for which only a few prerequisites are in place. These prerequisites include related virulent and avirulent strains, suitable suicide vectors for insertional inactivation, and an infection model for differentiation of virulent and avirulent strains. The described method should lead to the identification of relevant virulence determinants for a number of bacterial species and further the understanding of molecular pathogenesis.

ACKNOWLEDGMENTS

This work was supported by Department of Defense contract DAMD 17-98-C-8003, the Medical Research Council of Canada, and the Canadian Bacterial Diseases Network of Centers of Excellence. D.D. was a recipient of an Alberta Heritage Foundation for Medical Research (AHFMR) Fellowship award.

We thank Trish Darling and Amie White for excellent technical assistance. We thank Ivo Steinmetz for the EPS-specific monoclonal antibody 3015 and Malcolm Perry for many helpful discussions. We also thank E. Mahenthiralingam and P. Vandamme for sharing data prior to publication.

REFERENCES

1. **Antoine, R., and C. Locht.** 1992. Isolation and molecular characterization of a novel broad-host-range plasmid from *Bordetella bronchiseptica* with sequence similarities to plasmids from Gram-positive organisms. Mol. Microbiol. **6**:1785–1799.
2. **Blattner, F. R., G. Plunkett, III, C. A. Bloch, N. T. Perna, and V. Burland, et al.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277**:1453–1474.
3. **Borovkov, A. Y., and M. I. Rivkin.** 1997. *Xcm*-I containing vector for direct cloning of PCR products. BioTechniques **22**:812–814.
4. **Boulnois, G. J., and I. S. Roberts.** 1990. Genetics of capsular polysaccharide production in bacteria. Curr. Top. Microbiol. Immunol. **150**:1–18.
5. **Brett, P. J., and D. E. Woods.** 1996. Structural and immunologic characterization of *Burkholderia pseudomallei* O-polysaccharide–flagellin protein conjugates. Infect. Immun. **64**:2824–2828.
6. **Brett, P. J., D. DeShazer, and D. E. Woods.** 1997. Characterization of *Burkholderia pseudomallei* and *Burkholderia pseudomallei*-like strains. Epidemiol. Infect. **118**:137–148.
7. **Brett, P. J., D. DeShazer, and D. E. Woods.** 1998. *Burkholderia thailandensis* sp. nov., description of a *Burkholderia pseudomallei*-like species. Int. J. Syst. Bacteriol. **48**:317–320.
8. **Bryan, L. E., S. Wong, D. E. Woods, D. A. Dance, and W. Chaowagul.** 1994. Passive protection of diabetic rats with antisera specific for the polysaccharide portion of the lipopolysaccharide isolated from *Pseudomonas pseudomallei*. Can. J. Infect. Dis. **5**:170–178.
9. **Burtnick, M. N., and D. E. Woods.** 1999. Isolation of polymyxin B-susceptible mutants of *Burkholderia pseudomallei* and molecular characterization of genetic loci involved in polymyxin B resistance. Antimicrob. Agents Chemother. **43**:2648–2656.
10. **Chaiyaroj, S. C., K. Kotrnon, S. Koonpaew, N. Anantagool, N. J. White, and S. Sirisinha.** 1999. Differences in genomic macrorestriction patterns of arabinose-positive (*Burkholderia thailandensis*) and arabinose-negative *Burkholderia pseudomallei*. Microbiol. Immunol. **43**:625–630.
11. **Chaowagul, W., N. J. White, D. A. Dance, Y. Wattanagoon, P. Naigowit, T. M. Davis, S. Looareesuwan, and N. Pitakwatchara.** 1989. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. J. Infect. Dis. **159**:890–899.
12. **Clarke, B. R., R. Pearce, and I. S. Roberts.** 1999. Genetic organization of the *Escherichia coli* K10 capsule gene cluster: identification and characterization of two conserved regions in group III capsule gene clusters encoding polysaccharide transport functions. J. Bacteriol. **181**:2279–2285.
13. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, and C. Churcher, et al.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393**:537–544.
14. **Dance, D. A. B.** 1990. Melioidosis. Rev. Med. Microbiol. **1**:143–150.
15. **Darling, P., M. Chan, A. D. Cox, and P. A. Sokol.** 1998. Siderophore production by cystic fibrosis isolates of *Burkholderia cepacia*. Infect. Immun. **66**:874–877.
16. **DeShazer, D., P. J. Brett, R. Carlyon, and D. E. Woods.** 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn*5*-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J. Bacteriol. **179**:2116–2125.

17. **DeShazer, D., P. J. Brett, and D. E. Woods.** 1998. The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. Mol. Microbiol. **30**:1081–1100.
18. **DeShazer, D., and D. E. Woods.** 1999. Animal models of melioidosis, p. 199–203. *In* O. Zak and M. Sande (ed.), Handbook of animal models of infection. Academic Press, London, England.
19. **Falkow, S.** 1988. Molecular Koch's postulates. Rev. Infect. Dis. **10**:S274–S278.
20. **Frosch, M., U. Edwards, K. Bousset, B. Krausse, and C. Weisgerber.** 1991. Evidence for a common molecular origin of capsular gene loci in gram-negative bacteria expressing group II capsular polysaccharides. Mol. Microbiol. **5**:1251–1263.
21. **Frosch, M., D. Müller, K. Bousset, and A. Müller.** 1992. Conserved outer membrane protein of *Neisseria meningitidis* involved in capsule expression. Infect. Immun. **60**:798–803.
22. **Frosch, M., and A. Müller.** 1993. Phospholipid substitution of capsular polysaccharide and mechanisms of capsule formation in *Neisseria meningitidis*. Mol. Microbiol. **8**:483–493.
23. **Hacker, J., G. Blum-Oehler, I. Mühldorfer, and H. Tschäpe.** 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. **23**:1089–1097.
24. **Howe, C., A. Sampath, and M. Spotnitz.** 1971. The pseudomallei group: a review. J. Infect. Dis. **124**:598–606.
25. **Ip, M., L. G. Osterberg, P. Y. Chau, and T. A. Raffin.** 1995. Pulmonary melioidosis. Chest **108**:1420–1424.
26. **Karudapuram, S., X. Zhao, and G. J. Barcak.** 1995. DNA sequence and characterization of *Haemophilus influenzae dprA*$^+$, a gene required for chromosomal but not plasmid transformation. J. Bacteriol. **177**:3235–3240.
27. **Knirel, Y. A., N. A. Paramonov, A. S. Shashkov, N. K. Kochetkov, R. G. Yarullin, S. M. Farber, and V. I. Efremenko.** 1992. Structure of the polysaccharide chains of *Pseudomonas pseudomallei* lipopolysaccharides. Carbohydr. Res. **233**:185–193.
28. **Kroll, S., B. Loynds, L. Brophy, and E. Moxon.** 1990. The *bex* locus in encapsulated *Haemophilus influenzae*: a chromosomal region involved in capsule polysaccharide export. Mol. Microbiol. **4**:1853–1862.
29. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
30. **Leelarasamee, A., and S. Bovornkitti.** 1989. Melioidosis: review and update. Rev. Infect. Dis. **11**:413–425.
31. **Mack, K., and R. W. Titball.** 1998. The detection of insertion sequences within the human pathogen *Burkholderia pseudomallei* which have been identified previously in *Burkholderia cepacia*. FEMS Microbiol. Lett. **162**:69–74.
32. **Mahairas, G. G., P. J. Sabo, M. J. Hickey, D. V. Singh, and C. K. Stover.** 1996. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J. Bacteriol. **178**:1274–1284.
33. **Mahenthiralingam, E., T. Coenye, J. W. Chung, D. P. Speert, J. R. W. Govan, P. Taylor, and P. Vandamme.** 2000. Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. J. Clin. Microbiol. **38**:1042–1047.
34. **Mantsala, P., and H. Zalkin.** 1992. Cloning and sequence of *Bacillus subtilis purA* and *guaA*, involved in the conversion of IMP to AMP and GMP. J. Bacteriol. **174**:1883–1890.
35. **Masoud, H., M. Ho, T. Schollaardt, and M. B. Perry.** 1997. Characterization of the capsular polysaccharide of *Burkholderia pseudomallei* 304b. J. Bacteriol. **179**:5663–5669.
36. **Merriman, T. R., and I. L. Lamont.** 1993. Construction and use of a self-cloning promoter probe vector for Gram-negative bacteria. Gene **126**:17–23.
37. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170**:2575–2583.
38. **Mongkolsuk, S., S. Rabibhadana, P. Vattanaviboon, and S. Loprasert.** 1994. Generalized and mobilizable positive-selective cloning vectors. Gene **143**: 145–146.
39. **Moore, R. A., D. DeShazer, S. Reckseidler, A. Weissman, and D. E. Woods.** 1999. Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. Antimicrob. Agents Chemother. **43**:465–470.
40. **Nimtz, M., V. Wray, T. Domke, B. Brenneke, S. Haussler, and L. Steinmetz.** 1997. Structure of an acidic exopolysaccharide of *Burkholderia pseudomallei*. Eur. J. Biochem. **250**:608–616.
41. **Perry, M. B., L. L. MacLean, T. Schollaardt, L. E. Bryan, and M. Ho.** 1995. Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei*. Infect. Immun. **63**:3348–3352.
42. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493–497.
43. **Reeves, P. R., M. Hobbs, M. A. Valvano, M. Skurnik, C. Whitfield, D. Coplin, N. Kido, J. Klena, D. Maskell, C. R. Raetz, and P. D. Rick.** 1996. Bacterial polysaccharide synthesis and gene nomenclature. Trends Microbiol. **498**: 495–503.
44. **Roberts, I. S.** 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. Annu. Rev. Microbiol. **50**:285–315.
45. **Rocchetta, H. L., L. L. Burrows, J. C. Pacan, and J. S. Lam.** 1998. Three rhamnosyltransferases responsible for assembly of the A-band D-rhamnan polysaccharide in *Pseudomonas aeruginosa*: a fourth transferase, WbpL, is required for the initiation of both A-band and B-band lipopolysaccharide synthesis. Mol. Microbiol. **28**:1103–1119.
46. **Rosenow, C., F. Esumeh, I. S. Roberts, and K. Jann.** 1995. Characterization and localization of the KpsE protein from *Escherichia coli* K5, which is involved in polysaccharide export. J. Bacteriol. **177**:1137–1143.
47. **Sanford, J. P.** 1995. Pseudomonas species (including melioidosis and glanders), p. 2003–2009. *In* G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett (ed.), Principles and practice of infectious diseases. Churchill Livingstone, New York, N.Y.
48. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1**:784–791.
49. **Skorupski, K., and R. K. Taylor.** 1996. Positive selection vectors for allelic exchange. Gene **169**:47–52.
50. **Smith, A. N., G. J. Boulnois, and I. S. Roberts.** 1990. Molecular analysis of the *Escherichia coli* K5 kps locus: identification and characterization of an inner-membrane capsular polysaccharide transport system. Mol. Microbiol. **4**:1863–1869.
51. **Steinmetz, I., M. Rohde, and B. Brenneke.** 1995. Purification and characterization of an exopolysaccharide of *Burkholderia* (*Pseudomonas*) *pseudomallei*. Infect. Immun. **63**:3959–3965.
52. **Steinmetz, I., A. Reganzerowski, B. Brenneke, S. Haussler, A. Simpson, and N. J. White.** 1999. Rapid identification of *Burkholderia pseudomallei* by latex agglutination based on an exopolysaccharide-specific monoclonal antibody. J. Clin. Microbiol. **37**:225–228.
53. **Strauss, E. J., and S. Falkow.** 1997. Microbial pathogenesis: genomics and beyond. Science **276**:707–712.
54. **Trakulsomboon, S., D. A. B. Dance, M. D. Smith, N. J. White, and T. L. Pitt.** 1997. Ribotype differences between clinical and environmental isolates of *Burkholderia pseudomallei*. J. Med. Microbiol. **46**:565–570.
55. **Vandamme, P., B. Holmes, M. Vancanneyt, T. Coenye, B. Hoste, R. Coopman, H. Revets, S. Lauwers, M. Gillis, K. Kersters, and J. R. Govan.** 1997. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. Int. J. Syst. Bacteriol. **47**:1188–1200.
56. **Vandamme, P., E. Mahenthiralingam, B. Holmes, T. Coenye, B. Hoste, P. De Vos, D. Henry, and D. P. Speert.** 2000. Identification and population structure of *Burkholderia stabilis* sp. nov. (formerly *Burkholderia cepacia* genomovar IV). J. Clin. Microbiol. **38**:1042–1047.
57. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases, and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1**:945–951.
58. **Whitfield, C.** 1995. Biosynthesis of lipopolysaccharide O antigens. Trends Microbiol. **3**:178–185.
59. **Whitfield, C., and I. S. Roberts.** 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol. Microbiol. **31**:1307–1319.
60. **Wilson, K.** 1987. Preparation of genomic DNA from bacteria, p. 2.4.1–2.4.5. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
61. **Wunder, D. E., W. Aaronson, S. F. Hayes, J. M. Bliss, and R. P. Silver.** 1994. Nucleotide sequence and mutational analysis of the gene encoding KpsD, a periplasmic protein involved in transport of polysialic acid in *Escherichia coli* K1. J. Bacteriol. **176**:4025–4033.
62. **Wuthiekanun, V., M. D. Smith, D. A. Dance, A. L. Walsh, T. L. Pitt, and N. J. White.** 1996. Biochemical characteristics of clinical and environmental isolates of *Burkholderia pseudomallei*. J. Med. Microbiol. **45**:408–412.

Editor: V. J. DiRita

Microbiology (2001), 147, 000–000 Printed in Great Britain

Identification of the acid phosphatase (*acpA*) gene homologues in pathogenic and non-pathogenic *Burkholderia* spp. facilitates Tn*phoA* mutagenesis

Mary N. Burtnick, Alexandra J. Bolton, Paul J. Brett, David Watanabe and Donald E. Woods

Author for correspondence: Donald E. Woods. Tel: +1 403 220 2564. Fax: +1 403 283 5241. e-mail: woods@ucalgary.ca

University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada

Burkholderia pseudomallei and _Burkholderia mallei_ are pathogens responsible for disease in both humans and animals. _Burkholderia thailandensis_, while phylogenetically similar, is considered avirulent in comparison. These three species exhibit phosphatase activity when grown on media containing chromogenic substrates such as 5-bromo-4-chloro-3-indolyl phosphate (XP). Tn*5*-OT182 mutagenesis has been utilized to isolate mutants of _B. pseudomallei_ and _B. thailandensis_ unable to hydrolyse XP. Sequence analysis of these mutants revealed an ORF of 1734 nucleotides demonstrating a high degree of homology to the _acpA_ gene product of _Francisella tularensis_. PCR primers were designed based on the _B. pseudomallei acpA_ gene sequence and were used to amplify an _acpA_ homologue from _B. mallei_. The predicted amino acid sequence of _B. pseudomallei_ AcpA differed from those of the predicted _B. thailandensis_ AcpA and _B. mallei_ AcpA by 15 and 3 amino acids, respectively. Allelic exchange was used to construct Δ_acpA_ mutants in each of these _Burkholderia_ spp. These mutants were shown to be devoid of phosphatase activity and have subsequently allowed for the implementation of _phoA_ fusion transposon mutagenesis systems. Two such systems have been successfully utilized in _Burkholderia_ spp. for the identification of several genes encoding exported proteins.

Keywords: *Burkholderia* spp., acid phosphatase, Tn*phoA*, exported proteins

INTRODUCTION

Burkholderia pseudomallei, *Burkholderia thailandensis* and *Burkholderia mallei* are three closely related Gram-negative bacteria. *B. pseudomallei* is the causative agent of melioidosis, a disease endemic to South-east Asia and Northern Australia (Smith *et al.*, 1987) while *B. mallei* is the causative agent of glanders, an equine zoonosis (Arun *et al.*, 1999). *B. thailandensis* is an avirulent species that is genetically very similar to *B. pseudomallei* and *B. mallei*, however, it lacks at least one pathogenicity island present in these species (Brett *et al.*, 1998; Reckseidler *et al.*, 2000). Melioidosis and glanders have relatively high mortality rates and studies to elucidate the factors that contribute to their pathogenesis are necessary. *B. thailandensis* is a particularly useful laboratory tool for genetic manipulations under avirulent conditions which may contribute to the understanding of functions common to *B. pseudomallei* and *B. mallei*.

B. pseudomallei and *B. mallei* synthesize a variety of secreted enzymes (DeShazer *et al.*, 1999) and surface antigens; however, the roles of such factors in the pathogenesis of the diseases caused by these organisms remain poorly defined. To define the role(s) of particular exported proteins in pathogenesis, it is necessary to employ a system in which defined mutations can be made in genes encoding such products. The system we have chosen to investigate and implement in this study is the Tn*phoA* fusion vector system. The *phoA* gene fusion approach relies on the fact that the periplasmic bacterial

Abbreviations: AP, acid phosphatase; XP, 5-bromo-4-chloro-3-indolyl phosphate.

The GenBank accession numbers for the sequences reported in this paper are AF252862, AF252863 and AF275770.

alkaline phosphatase (PhoA) must be located extracytoplasmically for enzymic activity to occur (Taylor *et al.*, 1989; Manoil & Beckwith, 1985). Tn*phoA* utilizes a Tn5 transposon containing a truncated *phoA* gene which lacks a signal sequence; this transposon can generate *phoA* gene fusion randomly upon integration into the recipient bacterial chromosome (Taylor *et al.*, 1989; Manoil & Beckwith, 1985). If the targeted gene encodes an exported protein then the hybrid protein expressed will exhibit PhoA activity and the resulting colony will appear blue when grown on medium containing the chromogenic substrate 5-bromo-4-chloro-3-indoyl-phosphate (XP). Due to the fact that exported proteins are frequently involved in pathogenesis, this system provides a means by which the selection for the identification of virulence genes is enhanced. There are a number of instances in the literature in which Tn*phoA* mutagenesis has been used successfully for the identification of virulence factors. Some examples include involvement of OmpA in the virulence in *Escherichia coli* K-1 (Weiser & Gotschlich, 1991), identification of OMPs in the pathogenesis of *Salmonella abortusovis* (Rubino *et al.*, 1993), characterization of virulence genes of enteroinvasive *E. coli* (Hsia *et al.*, 1993), recognition that Tn*phoA* mutants in penicillin-binding proteins from *Erwinia amylovora* are avirulent (Milner *et al.*, 1993) and identification of antigens involved in colonization of *Vibrio cholerae* O139 (Bondre *et al.*, 1997).

B. pseudomallei exhibits phosphatase activity when grown on agar containing XP. To implement a *phoA* gene fusion system in *B. pseudomallei*, a strain that cannot hydrolyse XP must be utilized. It is known that some of this phosphatase activity is due to a surface-bound glycoprotein possessing acid phosphatase (AP) activity (Kanai & Kondo, 1994; Kondo *et al.*, 1996). However, the gene encoding an AP has remained unidentified prior to this study. In the present study we describe the sequence of the AP (*acpA*) gene homologues present in *B. pseudomallei*, *B. thailandensis* and *B. mallei*. The AP activity associated with the *acpA* gene product was assessed. The inactivation of the *acpA* gene homologues and subsequent complementation confirms that the *acpA* gene product is responsible for the AP activity present in these species. In addition, strains harbouring disrupted *acpA* gene homologues were constructed and have allowed for mutagenesis using Tn*phoA* (Manoil & Beckwith, 1985) and mini-O*phoA* (Bolton & Woods, 2000) for the identification of genes involved in the production of exported proteins in these *Burkholderia* spp.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *B. pseudomallei* and *B. thailandensis* cultures were incubated at 37 °C on Luria–Bertani (LB) agar plates or in LB broth with agitation at 250 r.p.m. *B. mallei* cultures were grown at 37 °C on tryptic soy agar supplemented with 4% glycerol (TSG) or in TSG broth. Antibiotics were purchased from Sigma and Invitrogen. For *E. coli*, antibiotics were used at the following concentrations: 100 μg ampicillin (Ap) ml^{-1}, 25 μg kanamycin (Km) ml^{-1}, 25 μg chloramphenicol (Cm) ml^{-1}, 100 μg streptomycin (Sm) ml^{-1}, 15 μg gentamicin (Gm) ml^{-1}, 15 μg tetracycline (Tc) ml^{-1}, 50 μg polymyxin B (Pm) ml^{-1}, 1·5 mg trimethoprim (Tp) ml^{-1} and 25 μg zeocin (Ze) ml^{-1}. For *B. pseudomallei* and *B. thailandensis* the antibiotic concentrations used were 50 μg Km ml^{-1}, 50 μg Tc ml^{-1}, 100 μg Tp ml^{-1} and 100 μg Ze ml^{-1} unless otherwise stated. For *B. mallei* antibiotic concentrations used were 75 μg naladixic acid (Nx) ml^{-1}, 5 μg Km ml^{-1}, 5 μg Ze ml^{-1}, 15 μg Pm ml^{-1} and 5 μg Gm ml^{-1}.

Plasmids were purified using the Concert rapid plasmid miniprep system (GibcoBRL), QIAprep spin plasmid miniprep kit (Qiagen) or QIAprep midipreps for plasmid DNA (Qiagen).

Tn5-OT182 mutagenesis and screening. *B. pseudomallei* 1026b and *B. thailandensis* E264 were mutagenized with Tn5-OT182 as previously described (DeShazer *et al.*, 1997). *B. pseudomallei* conjugations were incubated at 37 °C for 8 h while those of *B. thailandensis* were incubated at 37 °C for 12 h. Transconjugants were selected for on LB agar plates containing 100 μg Sm ml^{-1} and 50 μg Tc ml^{-1} with 40 μg XP ml^{-1}. White colonies were retained for further analyses.

DNA manipulation and transformations. Restriction endonucleases and T4 DNA ligase were purchased from GibcoBRL and New England Biolabs, respectively, and were used according to the manufacturers' instructions. DNA fragments excised from agarose gels and used in cloning procedures were purified using a QIAquick gel extraction kit (Qiagen). A Wizard genomic DNA purification kit (Promega) was used for isolation of genomic DNA from bacterial strains. The DNA immediately flanking Tn5-OT182 integrations was self-cloned as previously described (DeShazer *et al.*, 1997). In brief, approximately 5 μg chromosomal DNA from Tn5-OT182 mutants was digested with restriction enzyme, boiled for 5 min and precipitated with 0·1 vol. 3 M sodium acetate and 2 vols 100% ethanol. This mixture was placed at −70 °C for at least 30 min, centrifuged and washed with 70% ethanol. The resulting DNA was air-dried, resuspended in distilled water and ligation reactions were prepared. Transformations were performed with 2–10 μl ligation mixture using chemically competent *E. coli* cells.

Phosphatase activity assays. AP activity assays were performed in triplicate using a previously described method (Kondo *et al.*, 1991b, 1996). Supernatants, periplasmic proteins and whole cells were prepared from 1 ml of overnight cultures grown at 37 °C. Supernatants were harvested and filter-sterilized through a 0·22 μm filter (Millipore) for use in supernatant assays. Whole cells were pelleted, resuspended in 1 ml 0·01 M Tris/HCl pH 8·0 and used in whole-cell assays. Periplasmic proteins were extracted using a previously described chloroform extraction method (Ames *et al.*, 1984). In a typical assay, 20 μl of the test sample, 20 μl *p*-nitrophenyl phosphate (0·2%, w/v, solution) and 160 μl 0·1 M sodium acetate buffer pH 5·5 were mixed and incubated at 37 °C for 30 min in microtitre wells. Then 100 μl 0·5 M NaOH was added and the colour was allowed to develop for 5 min. Plates were read at 405 nm.

PCR amplification and cloning of PCR products. The *acpA* gene homologues were amplified from *B. pseudomallei* 1026b and *B. mallei* ATCC 23344 chromosomal DNA via PCR. The oligodeoxyribonucleotide primers used were AP-5′ (GCTCTAGACGAGCGGACGGGAAATGGCG)

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
E. coli		
DH5α	F⁻ ϕ80d*lacZ* ΔM15 Δ(*lacZYA–argF*)U169 *endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Bethesda Research Laboratories
Top 10	F⁻ *mcrA* Δ(*mrr–hsdRMS–mcrBC*) ϕ80*lacZ* ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara–leu*)7697 *galU galK rpsL* (Sm^R) *endA1 nupG*	Invitrogen
SM10 λ*pir*	SM10 with a λ prophage carrying the *pir* gene	Miller & Mekalanos (1988)
B. pseudomallei		
1026b	Clinical isolate (human); Km^R Gm^R Pm^R Sm^R Tc^S	DeShazer *et al.* (1997)
DD503	1026b derivative; allelic exchange strain; Δ(*amrR–oprA*); Km^S Gm^S Sm^S *rpsL* Sm^R	Moore *et al.* (1999)
APM402	1026b derivative; ORF::Tn*5*-OT182	This study
APM403	1026b derivative; *apcA*::Tn*5*-OT182	This study
APM403C	APM403 (p29*acpA*)	This study
MB401Z	DD503 derivative; *acpA*Δ(nt 1–1139)::*shble*-p15Aori; Ze^R	This study
MB401	DD503 derivative; *acpA* Δ(nt 1–1139)	This study
B. thailandensis		
E264	Environmental isolate; Km^R Gm^R Pm^R Sm^R Tc^S	Brett *et al.* (1998)
DW503	E264 derivative; allelic exchange strain; Δ(*amrR–oprA*); Km^S Gm^S Sm^S; *rpsL* Sm^R	This study
APM501	E264 derivative; *udp–rfaH*::Tn*5*-OT182	This study
APM502	E264 derivative; *apcA*::Tn*5*-OT182	This study
APM501C	APM501 (p29*acpA*)	This study
DW401Z	DW503 derivative; *acpA*Δ(nt 1–1139)::*shble*-p15Aori; Ze^R	This study
DW401	DW503 derivative; *acpA* Δ(nt 1–1139)	This study
B. mallei		
ATCC 23344 (formerly GB8)	Clinical isolate (equine)	USAMRIID
G8N	ATCC 23344 derivative; Nx^R	This study
G8P	ATCC 23344 derivative; *acpA*Δ(nt 1–1139)::*shble*-p15oriV; Ze^R	This study
G8PN	G8P derivative; Ze^R Nx^R	This study
Plasmids		
pOT182	pSUP102(Gm)::Tn*5*-OT182; Cm^R Gm^R Ap^R Tc^R	Merriman & Lamont (1993)
pUC19	Cloning vector	New England Biolabs
pCR2.1TOPO	Cloning vector; pUC ori; Ap^R Km^R	Invitrogen
pUCP29T	Broad-host-range vector; IncP OriT; pRO1600 ori; Tp^R	Schweizer *et al.* (1996)
p34EoriZeo	Vector containing self-cloning Ze^R cassette; *shble*-p15oriV; this vector was derived from p34Eori and the Zeo cassette from pEM7/Zeo (Invitrogen)	P. J. Brett, D. DeShazer, M. N. Burtnick & D. E. Woods, unpublished
pKAS46	Allelic exchange vector; *rpsL* Sm^S	Skorupski & Taylor (1996)
pAPM402E	0·7 kb *Eco*RI fragment from APM402 obtained by self-cloning; Ap^R Tc^R	This study
pAPM403E	8·0 kb *Eco*RI fragment from APM403 obtained by self-cloning; Ap^R Tc^R	This study
pAPM403H	5·5 kb *Hin*dIII fragment from APM403 obtained by self-cloning; Ap^R Tc^R	This study
pAPM501Ss	1·6 kb *Sst*I fragment from APM501 obtained by self-cloning; Ap^R Tc^R	This study
pAPM501H	6·5 kb *Hin*dIII fragment from APM501 obtained by self-cloning; Ap^R Tc^R	This study
pAPM502E	0·5 kb *Eco*RI fragment from APM502 obtained by self-cloning; Ap^R Tc^R	This study
pMB401	pUC19 containing 4·5 kb *Hpa*I/*Sst*I fragment containing nt 1–1633 of the *acpA* gene	This study
pMB401X	pMB401 Δ(2·8 kb *Xho*I fragment)	This study
pMB401Z	pMB401 Δ(2·8 kb *Xho*I fragment); *shble*-p15AoriV; Ze^R	This study
p46MB401X	pKAS46 containing 1·3 kb *Sst*I/*Hin*dIII fragment from pMB401X	This study
p46MB401Z	pKAS46 containing 2·7 kb *Sst*I/*Hin*dIII fragment from pMB401Z; Ze^R	This study
p29*acpA*	pUCP29T with 1·8 kb *Xba*I/KpnI fragment containing *B. pseudomallei acpA* gene	This study
pRT733	*oriR6K mobRP4* Tn*phoA*; Ap^R Km^R	Taylor *et al.* (1989)
pmini-O*phoA*	pMB1 *oriR*, Tn*5* *tnp*, RP4 *oriT*, *phoA*; Gm^R	A. J. Bolton & D. E. Woods, unpublished
pBR322	Cloning vector oriColE1; Ap^R Tc^R	New England Biolabs

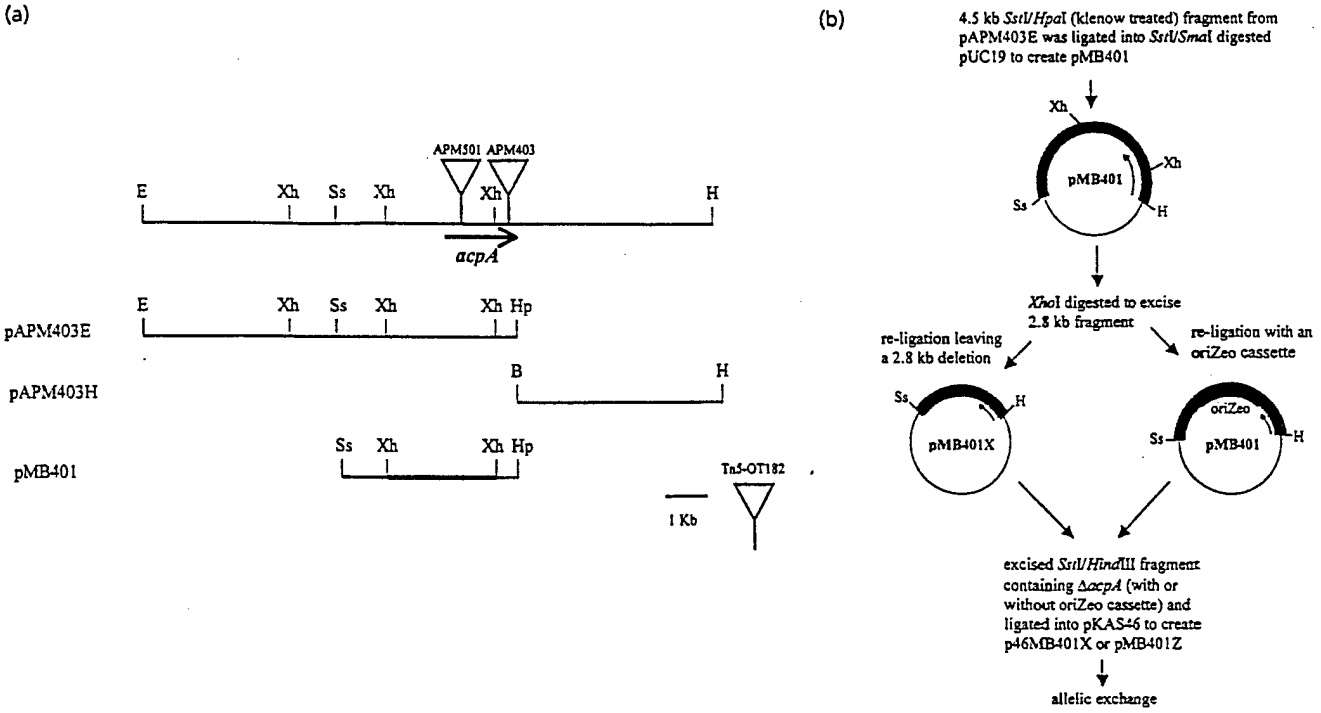


Fig. 1. (a) Plasmids containing *acpA* genes used in this study. Relative locations of Tn*5*-OT182 integrations in the *acpA* gene (black arrow) in AP-negative mutants, APM403 and APM501, are shown. Fragments obtained by self-cloning are shown, pAPM403E and pAPM403H. The fragment from pMB401 used for construction of strains for use in Tn*phoA* mutagenesis is also shown; the 2·8 kb fragment deleted encompassing nucleotides 1–1139 of the *acpA* gene is shown as a thicker black line. (b) Scheme used for deletion of a portion of the *B. pseudomallei acpA* gene (black arrow on inside of plasmid) and subsequent construction of vectors for allelic exchange. Restriction sites are as follows: *Eco*RI (E), *Hin*dIII (H), *Xho*I (Xh), *Sst*I (Ss), *Hpa*I (Hp) and *Bam*HI (B). The Hp and B sites were located on the ends of Tn*5*-OT182.

containing an *Xba*I linker and AP-3′ (GGGGTACCTCTT-GTCTACCGTACCGACC) containing a *Kpn*I linker (linkers underlined). PCR amplification was performed a 100 µl reaction mixtures containing approximately 500 ng genomic DNA, 1 × PCR buffer (GibcoBRL), a 200 mM concentration of each deoxynucleoside triphosphate, a 0·5 mM concentration of each primer, 2 mM $MgCl_2$ (GibcoBRL), 1 × Q-solution (Qiagen) and 5 U *Taq* DNA polymerase (GibcoBRL) per µl. This mixture was placed in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus) thermal cycler and subjected to a 5 min denaturation step at 95 °C followed by 30 cycles at 95 °C for 45 s, 56 °C for 30 s and 72 °C for 90 s. The reaction mixture was next held at 72 °C for 10 min and then placed at 4 °C until analysed on a 1 % agarose gel. The resulting PCR products were digested with *Kpn*I and *Xba*I and cloned into pUC19 or cloned directly into pCR2·1TOPO (Invitrogen) using a TOPO TA Cloning Kit (Invitrogen). The cloned PCR products were sequenced on both strands.

Construction of allelic exchange mutants. Allelic exchange was performed in *B. pseudomallei* DD503 and *B. thailandensis* DW503 using the *rpsL*-based vector pKAS46 as previously described (Moore *et al.*, 1999; Skorupski & Taylor, 1996). Both DD503 and DW503 are Sm^S due to deletion of the *amrR–oprA* operon, but Sm^R due to a mutation in the *rpsL* gene (Moore *et al.*, 1999). Allelic exchange experiments in the present study employed the vectors p46MB401Z or p46MB401X, which were constructed using a deleted version of the *acpA* gene homologue from *B. pseudomallei*. The steps in construction of these vectors are described below and are also shown in Fig. 1(b). A 4·5 kb *Sst*I/*Hpa*I (Klenow-treated) fragment containing nucleotides 1 to 1633 of the *acpA* gene was excised from pAPM403E and inserted into pUC19, creating pMB401. A 2·8 kb *Xho*I fragment was then excised from pMB401 and the vector was ligated back together with or without an oriZeo cassette, resulting in pMB401X or pMB401Z. Each of these fragments containing the Δ*acpA* gene homologue was separately inserted into pKAS46 to create p46MB401Z or p46MB401X. SM10 *λpir* strains containing these vectors were used in conjugation experiments with either DD503 or DW503. Transconjugants were selected for on LB agar containing 50 µg Pm ml^{-1}, 50 µg Km ml^{-1} and 100 µg Ze ml^{-1} when appropriate. Transconjugants were subsequently plated on 100 µg Sm ml^{-1} alone or with 100 µg Ze ml^{-1} to select for loss of the vector. These mutants were plated on LB agar plates containing 50 µg Km ml^{-1} to confirm a double crossover event as indicated by lack of growth. Allelic exchange mutants were confirmed by Southern blot analysis.

A slightly different strategy was used for allelic exchange in *B. mallei*, because spontaneous Sm^R mutants of ATCC 23344 were also Sm dependent, i.e. these mutants could not grow in the absence of Sm. To overcome this problem, transconjugants from ATCC 23344 and SM10 *λpir*(p46MB401Z) mutagenesis were selected for on TSG agar containing 80 µg XP ml^{-1}, 15 µg Pm ml^{-1} and 5 µg Ze ml^{-1}. One white transconjugant was Km^S, indicating loss of the vector (pKAS46) and thus, a double crossover event. This mutant was designated G8P. A spontaneously Nx^R derivative of G8P was selected for and obtained because Nx provided better selection than Pm. This Nx^R Ze^R XP-negative strain of *B. mallei* was designated G3PN and was used for further assessment.

Complementation of Tn5-OT182 mutants. A wild-type copy of the *acpA* gene obtained by PCR from *B. pseudomallei* 1026b DNA was cloned into the broad-host-range vector pUCP29T. This construct, designated p29*acpA*, was transformed into *E. coli* SM10 *λpir* and conjugated to *B. pseudomallei* APM403 and *B. thailandensis* APM501 for 5 h, followed by selection on LB agar plates containing 100 μg Sm ml^{-1} and 100 μg Tp ml^{-1}. The resulting strains were inoculated on similar LB agar plates with 40 μg XP ml^{-1} and blue colonies were retained.

TnphoA and mini-OphoA mutagenesis. In a typical TnphoA mutagenesis experiment, approximately 5 μl of an overnight culture of SM10 *λpir*(pRT733) containing TnphoA and 5 μl of either *B. pseudomallei* MB401Z or *B. thailandensis* DW401Z were mixed together on an LB agar plate and incubated at 37 °C for 18 h. Eight to ten separate conjugations were carried out on a single plate concurrently along with donor and recipient alone as controls. Each individual conjugation was plated on a single agar plate. *B. pseudomallei* and *B. thailandensis* transconjugants were selected for on LB agar containing 300 μg Km ml^{-1}, 100 μg Sm ml^{-1}, 100 μg Ze ml^{-1} and 40 μg XP ml^{-1}. For *B. mallei* G8PN a similar procedure was employed except that transconjugants were selected for on TSG agar plates containing 5 μg Km ml^{-1}, 75 μg Nx ml^{-1}, 5 μg Ze ml^{-1} and 80 μg XP ml^{-1}. Plates were incubated at 37 °C for 48 h and blue colonies were retained for further analysis. The DNA immediately flanking the TnphoA integration was cloned as previously described (Taylor *et al.*, 1989) using the cloning vector pBR322 and *Bam*HI- or *Sal*I-digested genomic DNA. The resulting plasmids were sequenced using a previously described primer sequence (Taylor *et al.*, 1989)

Mini-OphoA was constructed using the Tn5-based plasposon pTnModOGm (Dennis & Zylstra, 1998) and the *phoA* gene from pRT733 (TnphoA) (Manoil & Beckwith, 1985). Mini-OphoA is small (3·4 kb) and contains an origin of replication that allows for self-cloning of the chromosomal DNA adjacent to transposon integrations (Bolton & Woods, 2000). *B. pseudomallei* MB401, *B. thailandensis* DW401 and *B. mallei* G8PN strains were recipient strains for mini-OphoA mutagenesis experiments. Conjugations were performed as described for TnphoA using 5 μl of donor and recipient strains on LB or TSG agar plates at 37 °C for 18 h. Transconjugants of *B. pseudomallei* and *B. thailandensis* were selected for on LB agar plates containing 100 μg Sm ml^{-1}, 15 μg Gm ml^{-1} and 40 μg XP ml^{-1}. *B. mallei* transconjugants were selected for on TSG agar plates containing 5 μg Gm ml^{-1}, 75 μg Nx ml^{-1}, 5 μg Ze ml^{-1} and 80 μg XP ml^{-1}. Self-cloning of the DNA immediately flanking mini-OphoA integrations was performed essentially as previously described for Tn5-OT182 (DeShazer *et al.*, 1997). Briefly, genomic DNA of mutants harbouring mini-OphoA was isolated then digested with *Not*I at 37 °C for 1 h. These reactions were then heat-inactivated followed by ethanol precipitation. Ligation reactions were set up for 1 h at room temperature or overnight at 16 °C then transformed into chemically competent *E. coli* DH5α or Top 10 cells. The resulting plasmids were isolated and sequenced.

DNA sequencing and analysis. DNA sequencing was performed by University Core DNA Services (University of Calgary). The previously described oligodeoxyribonucleotide primers OT182-RT and OT182-LT (DeShazer *et al.*, 1997) were used for sequencing of plasmid DNA obtained by self-cloning of Tn5-OT182 mutants. The previously described primer sequence (5′-AATATCGCCCTGAGC-3′) was used for sequencing plasmids from TnphoA clones obtained in this study (Taylor *et al.*, 1989). Two deoxyoligonucleotide primers, Pho-LT (5′-CAGTAATATCGCCCTGAGCAGC-3′) and Gm-RT (5′-GCCGCGCAATTCGAGCTC-3′), were used for sequencing the mini-OphoA clones (Bolton & Woods, 2000). Custom-designed primers were synthesized by University Core DNA Services and used in a primer walking strategy to obtain the sequence of both strands of the *acpA* gene homologue.

The DNA sequences obtained in this study were analysed using DNASIS v2.5 (Hitachi) and DIALIGN 2.1 (Morgenstern, 1999) for the presence of ORFs and restriction endonuclease cleavage sites, for sequence alignment and for translation to amino acid sequences. BLASTX and BLASTP programs were used to perform database searches in order to establish homology to known gene sequences (Altschul *et al.*, 1997).

The *acpA* gene sequences from *B. pseudomallei*, *B. thailandensis* and *B. mallei* were submitted to GenBank under accession nos AF252862, AF252863 and AF276770, respectively.

RESULTS

Identification of *acpA* homologues from *B. pseudomallei*, *B. thailandensis* and *B. mallei*

To identify and characterize the gene or genes responsible for the phosphatase activity exhibited by *B. pseudomallei*, we chose to employ Tn5-OT182 mutagenesis in combination with a simple screen in which the chromogenic substrate XP was incorporated into LB agar plates. Approximately 7000 *B. pseudomallei* Tn5-OT182 mutants were plated onto media containing XP. Two mutants were identified that were unable to hydrolyse XP as indicated by their lack of blue colour. These two *B. pseudomallei* phosphatase-negative mutants were designated APM402 and APM403. The DNA flanking the Tn5-OT182 integrations in each of these mutants was isolated by self-cloning using *Eco*RI and *Hin*dIII. The resulting plasmids were isolated and single-stranded sequence reactions were carried out. The sequences obtained were analysed using the BLASTX local alignment search tool. The sequence from APM403 demonstrated a high degree of homology to the AP of *Francisella tularensis* var. *novicida*. In contrast, the sequence from APM402 showed no significant homology to any sequences currently in the GenBank database.

The self-cloned plasmids, pAPM403E and pAPM403H (Fig. 1a), were sequenced for approximately 2 kb on each side of the Tn5-OT182 integration on both strands using a primer walking strategy. An ORF of 1734 nucleotides was identified. The product of this ORF demonstrated 36% similarity to the *acpA* gene of *F. tularensis* and was therefore designated the *B. pseudomallei acpA* gene homologue. PCR primers were designed based on this sequence in order to identify the *acpA* gene homologues of *B. thailandensis* and *B. mallei*, both of which exhibit phosphatase activity. This approach was successful for identification of the *B. mallei acpA* homologue, but ineffective for identifying the *B. thailandensis acpA* homologue. The PCR product obtained from *B. mallei* ATCC 23344 chromosomal DNA using the AP-5′ and AP-3′ primers migrated to the

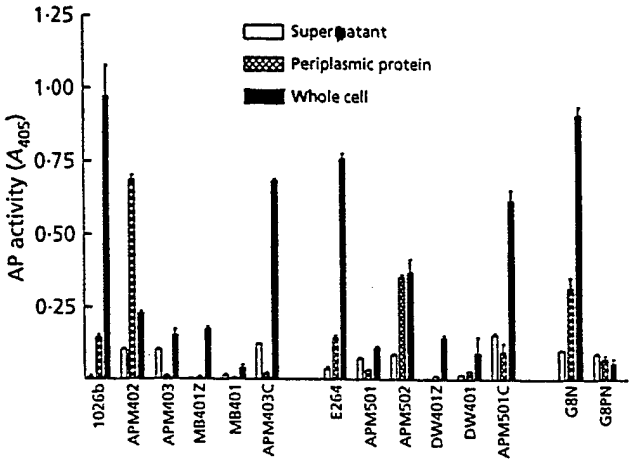


Fig. 2. AP activities of the *B. pseudomallei*, *B. thailandensis* and *B. mallei* strains used in this study. Supernatant, periplasmic and whole-cell fractions were prepared from overnight cultures grown at 37 °C. See Methods for details. The values are means and standard deviations of a single experiment performed in triplicate.

same position as the *B. pseudomallei* 1026b PCR product on a 1% agarose gel; both were approximately 1·8 kb in size. This result indicated that the *acpA* gene homologues present in both *B. pseudomallei* and *B. mallei* were probably very similar. These products were cloned and subjected to sequence analysis, which confirmed them to be *acpA* homologues. The sequence of the *B. mallei acpA* homologue was then completed on both strands.

Since we were unable to obtain a *B. thailandensis acpA* homologue by PCR, we chose to employ Tn*5*-OT182 mutagenesis to isolate this gene. Approximately 5000 Tn*5*-OT182 mutants of *B. thailandensis* E264 were plated onto LB agar plates containing XP and two white mutants were obtained. These mutants were designated APM501 and APM502. The chromosomal DNA immediately flanking the Tn*5*-OT182 integrations in these mutants was obtained by self-cloning using *Sst*I and *Hin*dIII. The sequences obtained from pAPM501Ss and pAPM501H demonstrated highest homology to the *B. pseudomallei acpA* gene homologue. Primer walking was employed to sequence the *B. thailandensis acpA* homologue on both strands. The sequence from the *B. thailandensis* APM502 was shown to have highest homology to the UDP-*rfaH* intergenic region of *E. coli*.

AP activity of *B. pseudomallei*, *B. thailandensis* and *B. mallei* strains

AP activity has previously been characterized for *B. pseudomallei* and it has been shown that optimal activity occurs at pH 5·5 and at 37 °C (Kanai & Kondo, 1994; Kondo *et al.*, 1991a, 1996). To confirm that the mutant strains isolated in this study lacked any AP activity, both the parent strains and the mutant strains were assayed as described in Methods (Fig. 2). All three parent strains, *B. pseudomallei* 1026b, *B. thailandensis* E264 and *B. mallei* ATCC 23344, demonstrated similar levels of AP activity. The results of this assay indicated that the Tn*5*-OT182 mutants, APM403 and APM501, lacked any observable AP activity. In contrast, APM402 had considerable AP activity restricted to the periplasmic fraction and APM502 retained observable amounts of AP activity in both the periplasmic and whole-cell fractions.

Nucleotide sequence analysis of the *acpA* genes of *B. pseudomallei*, *B. thailandensis* and *B. mallei*

The nucleotide sequences of the *acpA* gene homologues from *B. pseudomallei* 1026b, *B. thailandensis* E264 and *B. mallei* ATCC 23344 demonstrated 23% identity and 36% similarity with the *acpA* gene product of *F. tularensis*. A 1734 bp ORF was identified, beginning with the ATG codon and ending with the TGA codon, that was consistent for *B. pseudomallei* and *B. mallei*, while a GTG start codon was identified for *B. thailandensis*. The G+C content of the *acpA* ORF was determined to be 69 mol%. A putative Shine–Dalgarno sequence was identified −6 to −10 bp upstream of the putative ATG/GTG start codons, suggesting that these are the correct start sites. The nucleotide sequences obtained for *B. thailandensis* and *B. mallei* were 94% and 99% identical, respectively, to the *B. pseudomallei acpA* sequence. The Tn*5*-OT182 integration associated with APM403 occurred at nucleotide 1633 of the *acpA* ORF while the Tn*5*-OT182 integration associated with APM501 occurred at nucleotide 365. The approximate positions are shown in Fig. 1(a). The putative protein encoded by *acpA* was predicted to be 578 amino acids in length with a calculated molecular mass of 62860 Da. Comparison of the predicted amino acid sequences of *B. thailandensis* AcpA and *B. mallei* AcpA to that of the *B. pseudomallei* AcpA predicted amino acid sequence revealed 15 and 3 differences, respectively. This reflects the close phylogenetic relationship between these species.

Characterization of AP-negative allelic exchange mutants

AP-negative mutants were constructed by allelic exchange as previously described with *B. pseudomallei* DD503 and *B. thailandensis* DW503 using the vectors p46MB401Z or p46MB401X (Fig. 1b). These vectors contained a portion of the *acpA* gene including nucleotides 1140–1633 along with 1·5 kb of upstream DNA that has not yet been sequenced. The *B. pseudomallei* Δ*acpA* mutants were designated MB401Z and MB401 and the *B. thailandensis* Δ*acpA* mutants were designated DW401Z and DW401. The strains MB401 and DW401 have a deletion in their *acpA* genes and lack the oriZeo marker present in the other AP-negative allelic exchange mutants, thus eliminating the need for the presence of Ze in selective media. As described in Methods, a slightly different strategy using the p46MB401Z vector was employed for allelic exchange in *B. mallei*. The

oriZeo cassette was used for positive selection in *B. mallei* allelic exchange and Ze was used in further experiments. The resulting Δ*acpA* strain of *B. mallei* was designated G8PN and was assessed for AP activity.

The isogenic allelic exchange mutant strains MB401Z/MB401, DW401Z/DW401 and G8PN were unable to hydrolyse the chromogenic substrate XP when present in LB or TSG agar. This was consistent with the observation that Tn*5*-OT182 disruptions in the *acpA* homologue caused APM403 and APM501 to display a white phenotype. Additionally, the allelic exchange mutants were essentially devoid of AP activity at pH 5·5 (Fig. 2) compared to wild-type strains. The inability of these strains to display blue colour when grown on agar containing XP made them good candidates as recipients for mutagenesis with Tn*phoA* and thus for the identification of exported products.

Complementation of AP-negative Tn*5*-OT182 strains

The 1·8 kb PCR product harbouring the *acpA* gene homologue *B. pseudomallei* was cloned into pUCP29T and was conjugated to *B. pseudomallei* APM403 and *B. thailandensis* APM501. The presence of p29*acpA* was able to restore the AP activity of these strains. The complemented strains, designated APM403C and APM501C, were blue when grown on LB agar plates containing XP and exhibited activity by AP activity assay (Fig. 2). These results indicate that the AP-negative phenotype observed in APM403 and APM501 is due to the Tn*5*-OT182 disruption in their *acpA* gene homologues and that this gene encodes a product that is responsible for the AP activity observed in these organisms. The strains constructed for Tn*phoA* mutagenesis were not complemented as the mutation encompasses 2·8 kb that has not been completely sequenced.

Tn*phoA* and mini-O*phoA* mutagenesis of *B. pseudomallei*, *B. thailandensis* and *B. mallei*

Two Tn*5*-based transposons containing truncated *phoA* genes were employed in this study. Initially, Tn*phoA* was delivered to MB401Z, DW401Z and G8PN on the vector pRT733 as previously described (Taylor *et al.*, 1989). This system worked efficiently for *B. pseudomallei* and *B. thailandensis*, resulting in approximately 1000–1200 Sm^R Km^R transconjugants per mutagenesis experiment, 1% of which were PhoA positive. However, in *B. mallei*, the Tn*phoA* transposition frequency was significantly lower: each mutagenesis resulted in only 50–200 Nx^R Km^R transconjugants with a frequency of PhoA-positive colonies of approximately 2%. Southern blot analysis using *Bam*HI-digested chromosomal DNA from Tn*phoA* mutants confirmed that Tn*phoA* integrated only once per chromosome in four randomly selected *B. pseudomallei* and *B. thailandensis* PhoA-positive mutants.

Although this system is functional in these strains, the cloning procedures had a low efficiency, approximately 25%. This is suspected to be due in part to the size of the transposon and the fact that the cloning vector, pBR322, has a size limit on the DNA inserts that it can efficiently accept (approx. 7 kb). Upon digestion of the chromosomal DNA of PhoA-positive mutants with *Bam*HI or *Sal*I at least 5 kb of transposon remains along with the chromosomal DNA immediately flanking fragment. The cloning of DNA fragments containing Tn*phoA* and adjacent chromosomal DNA into pBR322 resulted in only relatively small (< 2 kb) flanking DNA sequences being obtained. The resulting plasmids were sequenced and BLASTX searches were performed. Sequences showing significant homology over at least 300 bp of flanking DNA are shown in Table 2.

Due to the low numbers of transconjugants in *B. mallei* Tn*phoA* mutagenesis experiments, we chose to employ a second transposon, designated mini-O*phoA* (Bolton & Woods, 2000). The mini-O*phoA* system was found to be equivalent in transposition frequency to Tn*phoA* in *B. pseudomallei* and *B. thailandensis*, with the occurrence of PhoA-positive colonies being 0·5%. The transposition frequency was increased in *B. mallei*: approximately 1500 Ze^R Nx^R Gm^R transconjugants were obtained per mutagenesis, with the frequency of PhoA-positive colonies being 2%. In addition, due to the presence of an origin of replication, the cloning procedures for obtaining the DNA sequences flanking mini-O*phoA* integrations were simpler and more efficient than those for Tn*phoA*. Cloning efficiency was 90% when chemically competent *E. coli* Top 10 cells (Invitrogen) were used. The chromosomal DNA of six random Sm^R Gm^R transconjugants of *B. pseudomallei* and *B. thailandensis* and three random Ze^R Nx^R Gm^R transconjugants of *B. mallei* was isolated, digested with *Not*I and probed with α-^{32}P-labelled mini-O*phoA*. All nine mutants contained single copies of this transposon, suggesting that mini-O*phoA* integrates randomly into the chromosomes of *B. pseudomallei*, *B. thailandensis* and *B. mallei*.

The DNA from a number of PhoA-positive *B. pseudomallei*, *B. thailandensis* and *B. mallei* mini-O*phoA* mutants was self-cloned and subjected to single-stranded sequencing in order to characterize the DNA flanking the transposon integrations. Approximately 500–700 bp of sequence was obtained on each side of the mini-O*phoA* integrations. Subsequently, database searches were performed in order to establish homologies to known gene sequences. Some of the sequences obtained from the Pho-LT primer demonstrated significant homology over at least 300 bp and are shown in Table 2. A number of putative genes were identified which encoded proteins showing homology to secreted proteins, confirming the ability of this system to identify extracytoplasmic products expressed by the three *Burkholderia* spp. utilized in this study.

The DNA sequences adjacent to Tn*phoA* and mini-O*phoA* integrations in a number of PhoA-positive mutants did not show any significant homology to sequences currently in the GenBank database. These

Table 2. Table of homology of Tn*phoA* and mini-O*phoA* flanking sequences

PhoA-positive mutant	Identity (%)	Similarity (%)	Homology	Entrez protein ID
B. pseudomallei Tn*phoA* mutants				
PHOA8	40	43	Putative cell wall protein of *Streptomyces coelicolor*	AL137165
PHOA16	99	99	*gspG* (*B. pseudomallei*) type II secretion pathway gene	AAD05177.1
PHOA20	23	33	Hydroxyproline-rich glycoprotein of *Zea diploperennis*	228938
PHOA39	31	38	ExiT protein (exochelin ABC transporter) from *Mycobacterium smegmatis*	AAC32046.1
PHOA47	32	50	Outer-membrane protein C of *Pseudomonas aeruginosa*	BAA05664.1
B. mallei Tn*phoA* mutant				
AJB34	75	95	Dipeptide transport system permease protein of *Escherichia coli*	AAC76568.1
B. pseudomallei mini-O*phoA* mutants				
PHOG4	33	43	Penicillin-binding protein of *Deinococcus radiodurans*	AAF10059.1
PHOG9	45	63	Periplasmic serine protease from *Aquifex aeolicus*	AAC07399.1
PHOG18	52	76	Putrescine-binding periplasmic protein precursor; permease protein from *Escherichia coli*	AAC73941.1
PHOG28	69	84	Phosphate-binding periplasmic protein precursor of *Erwinia carotovora*	AAB70458.1
PHOG29	37	50	Outer-membrane porin protein OpcP1 of *Burkholderia cepacia*	BAA09892.1
B. thailandensis mini-O*phoA* mutants				
PHOG103	36	44	Putative YME1 ATP-dependent zinc protease of *Arabidopsis thaliana*	AAC31223.1
B. mallei mini-O*phoA* mutant				
AJB101	43	56	Probable ABC transporter, permease protein of *Treponema pallidum*	F71375
AJB116	40	60	Branched-chain amino acid ABC transporter of *Deinococcus radiodurans*	H75444
AJB139	69	80	Phosphate-binding protein of *Enterobacter cloacae*	BAA22861.1
AJB150	36	50	Putative aromatic efflux pump outer-membrane protein of *Sphingomonas aromaticivorans*	AAD03862.1
AJB153	60	73	Periplasmic sorbitol-binding protein of *Rhodobacter sphaeroides*	AAC45766.1
AJB171	63	75	Outer-membrane protein C of *Pseudomonas aeruginosa*	BAA05664.1

sequences are of significant interest and may represent as yet undefined genes encoding exported products.

DISCUSSION

The AP activity of *B. pseudomallei* has been previously documented (Kanai & Kondo, 1994; Kondo *et al.*, 1991a, b, 1996); however, the gene responsible for this activity had not been identified prior to this study. The present work has demonstrated the presence of *acpA* gene homologues in *B. pseudomallei*, *B. thailandensis* and *B. mallei*. A simple screen was employed to identify mutants devoid of phosphatase activity. Several Tn*5*-OT182 mutants unable to hydrolyse XP were isolated and these subsequently facilitated the identification of *acpA* gene homologues in both *B. pseudomallei* and *B. thailandensis*. PCR primers designed from the nucleotide sequence of the *B. pseudomallei acpA* gene allowed the identification of an *acpA* homologue from *B. mallei*. The *acpA* genes from the species used in this study were sequenced and the predicted amino acid compositions reflected the close phylogenetic relationship between these species. Complementation analyses have shown that functional *acpA* gene homologues are required for AP activity in these three *Burkholderia* spp. Furthermore, identification of *acpA* genes in these species has allowed the construction of strains with Δ*acpA* genes through allelic exchange.

The AP-negative strains constructed in this study have been used for mutagenesis experiments employing Tn*5*-based transposons containing truncated *phoA* genes. The *B. thailandensis* strain DW401/DW401Z will be particularly useful as it is a non-virulent strain that can be used as a laboratory tool for the identification of genes likely to be present in the highly virulent, closely related *B. pseudomallei* and *B. mallei* strains. The results of this study clearly indicate that Tn*5*-based transposons containing truncated *phoA* genes can be efficiently used in *B. pseudomallei* and *B. thailandensis* strains. It is not clear why Tn*phoA* mutagenesis was not effective in *B.*

mallei; it may be due to an incompatibility with the vector carrying the transposon. However, this problem was overcome by employing a second transposon system, mini-O*phoA*, that was shown to integrate efficiently in this species.

PhoA-positive transposon mutants have been isolated in this study and sequence analysis of DNA flanking transposon insertions has revealed homology to a number of known gene sequences. We have demonstrated that the *phoA* fusion approach can be efficiently used in *Burkholderia* spp. for the identification of genes encoding exported proteins. The *phoA* systems employed in this study have facilitated the identification of genes potentially contributing to the pathogenesis of melioidosis and glanders. We are currently constructing isogenic mutants in specific genes identified via *phoA* mutagenesis; this will allow for the assessment of the contribution of particular genes to the phenotypes displayed by these organisms. Such mutants will be used in virulence testing. This will help to establish the roles specific exported products play in pathogenesis.

Preliminary studies on the role of the *acpA* gene product in the pathogenesis of *B. pseudomallei* and *B. mallei* infections indicate that the disruption of the *acpA* gene does not significantly alter virulence (data not shown). The mutants harbouring disrupted *acpA* genes may be useful for future studies regarding the specific functioning of the *acpA* gene and its product. Identification of the *acpA* gene and the subsequent implementation of *phoA* mutagenesis systems described in the present study will contribute to the continuing studies on the pathogenesis of melioidosis and glanders.

ACKNOWLEDGEMENTS

This research is supported by the Canadian Bacterial Diseases Network Centers for Excellence. M. N. B. is the recipient of an Alberta Heritage Foundation for Medical Research (AHFMR) studentship. D. W. is the recipient of an AHFMR summer studentship.

REFERENCES

Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25, 3389–3402.

Ames, G. F., Prody, C. & Kustu, S. (1984). Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *J Bacteriol* 160, 1181–1183.

Arun, S., Neubauer, H., Gurel, A., Ayyildiz, G., Kuscu, B., Yesildere, T., Meyer, H. & Hermanns, W. (1999). Equine glanders in Turkey. *Vet Rec* 144, 255–258.

Bolton, A. J. & Woods, D. E. (2000). A self-cloning minitransposon *phoA* gene fusion system promotes the rapid analysis of secreted proteins in Gram negative bacteria. *Biotechniques* 29, 470–474.

Bondre, V. P., Srivastava, R., Sinha, V. B. & Srivastava, B. S. (1997). Screening of TnphoA mutants of *Vibrio cholerae* O139 for identification of antigens involved in colonisation. *J Med Microbiol* 46, 1007–1011.

Brett, P. J., DeShazer, D. & Woods, D. E. (1998). *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. *Int J Syst Bacteriol* 48, 317–320.

Dennis, J. J. & Zylstra, G. J. (1998). Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. *Appl Environ Microbiol* 64, 2710–2715.

DeShazer, D., Brett, P. J., Carlyon, R. & Woods, D. E. (1997). Mutagenesis of *Burkholderia pseudomallei* with Tn*5*-OT182: isolation of motility mutants and molecular characterisation of the flagellin structural gene. *J Bacteriol* 179, 2116–2125.

DeShazer, D., Brett, P. J., Burtnick, M. N. & Woods, D. E. (1999). Molecular characterisation of genetic loci required for secretion of exoproducts in *Burkholderia pseudomallei*. *J Bacteriol* 181, 4661–4664.

Hsia, R. C., Small, P. L. & Bavoil, P. M. (1993). Characterisation of virulence genes of enterinvasive *Escherichia coli* by Tn*phoA* mutagenesis: identification of *invX*, a gene required for entry into HEp-2 cells. *J Bacteriol* 175, 4817–4823.

Kanai, K. & Kondo, E. (1994). Recent advances in biomedical sciences of *Burkholderia pseudomallei* (basonym: *Pseudomonas pseudomallei*). *Jpn J Med Sci Biol* 47, 1–45.

Kondo, E., Dejsirilert, S., Wejprasit, N., Chiewsilp, D. & Kanai, K. (1991a). Heat-stable and heat-labile components of nonspecific acid phosphatase detected in *Pseudomonas pseudomallei*. *Jpn J Med Sci Biol* 44, 51–62.

Kondo, E., Petkanchanapong, V., Naigowit, P., Kurata, T. & Kanai, K. (1991b). Demonstration of acid phosphatase activity in antigenic glycoprotein fractions obtained from the culture filtrate of *Pseudomonas pseudomallei*. *Jpn J Med Sci Biol* 44, 213–224.

Kondo, E., Kurata, T., Naigowit, P. & Kanai, K. (1996). Evolution of cell-surface acid phosphatase of *Burkholderia pseudomallei*. *Southeast Asian J Trop Med Public Health* 27, 592–599.

Manoil, C. & Beckwith, J. (1985). Tn*phoA*: a transposon probe for protein export signals. *Proc Natl Acad Sci USA* 82, 8129–8133.

Merriman, T. R. & Lamont, I. L. (1993). Construction and use of a self-cloning promoter probe vector for gram-negative bacteria. *Gene* 126, 17–23.

Miller, V. L. & Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* 170, 2575–2583.

Milner, J. S., Dymock, D., Cooper, R. M. & Roberts, I. S. (1993). Penicillin-binding proteins from *Erwinia amylovora*: mutants lacking PBP2 are avirulent. *J Bacteriol* 175, 6802–6208.

Moore, R. A., DeShazer, D., Reckseidler, S. L., Weissman, A. & Woods, D. E. (1999). Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. *Antimicrob Agents Chemother* 43, 465–470.

Morgenstern, B. (1999). DIALIGN 2: improvement of the segment-to-segment approach to multiple sequence alignment. *Bioinformatics* 15, 211–218.

Reckseidler, S. L., DeShazer, D., Sokol, P. A. & Woods, D. E. (2000). Detection of bacterial virulence genes by subtractive hybridisation: identification of the capsular polysaccharide of *Burkholderia pseudomallei* as a major virulence determinant. *Infect Immun* 00, 0000–0000.

Rubino, S., Leori, G., Rizzu, P., Erre, G., Colombo, M. M., Uzzau, S., Masala, G. & Cappuccinelli, P. (1993). Tn*phoA* *Salmonella abortusovis* mutants unable to adhere to epithelial cells with reduced virulence in mice. *Infect Immun* 61, 1786–1792.

Schweizer, H., Klassen, T. & Hoang, T. (1996). Improved methods for gene analysis and expression in *Pseudomonas* spp. In

Molecular Biology of Pseudomonads, pp. 000–000. Edited by T. Nakazawa, K. Furukawa, D. Haas & S. Silver. Washington, DC: American Society for Microbiology.

Skorupski, K. & Taylor, R. K. (1996). Positive selection vectors for allelic exchange. *Gene* 169, 47–52.

Smith, C., Allen, J., Embi, M., Othman, O., Razak, N. & Ismail, G. (1987). Human melioidosis: an emerging medical problem. *MIRCEN J Appl Microbiol Biotechnol* 3, 343–366.

Taylor, R. K., Manoil, C. & Mekalanos, J. J. (1989). Broad-host-range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. *J Bacteriol* 171, 1870–1878.

Weiser, J. N. & Gotschlich, E. C. (1991). Outer membrane protein A (OmpA) contributes to serum resistance and pahtogenicity of *Escherichia coli* K-1. *Infect Immun* 59, 2252–2258.

Received 27 June 2000; revised 12 September 2000; accepted 5 October 2000.

Self-Cloning Minitransposon *phoA* Gene-Fusion System Promotes the Rapid Genetic Analysis of Secreted Proteins in Gram-Negative Bacteria

BioTechniques 29:470-474 (September 2000)

Virulence determinants are inclined to be located on the bacterial surface or secreted into the milieu where they can interact with the host. A system that isolates defined mutations in secreted bacterial products is an invaluable tool for identifying virulence genes. One such system is the *phoA* gene-fusion approach that takes advantage of the fact that the normally periplasmic bacterial protein alkaline phosphatase (PhoA) is only biologically functional when secreted from the cytoplasm (2,4). Therefore, when a truncated form of *phoA*, lacking its signal sequence, inserts downstream of and in-frame with a signal sequence and forms a gene fusion, the exported fusion protein exhibits phosphatase activity.

Isolation of mutants carrying active PhoA-fusions is a simple procedure: colonies appear blue when grown on agar plates incorporated with the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The most widely used *phoA*-fusion system is the Tn*phoA* transposon mobilized onto broad host range suicide plasmids such as pRT733 (4). Because of the broad host range of both delivery plasmids and Tn5 transposon, this system is applicable to a wide range of Gram-negative bacterial species. However, Tn*phoA* lacks convenient cloning sites, which results in the inefficient isolation of clones containing DNA flanking the transposon, and the isolated DNA is often composed of small segments. In addition, the presence of the cognate transposase within the inverted repeats can confer instability upon the transposon and induce further DNA rearrangements.

Here, we describe the construction of a Gram-negative, broad host range self-cloning minitransposon *phoA* gene-fusion system that markedly increases the efficiency of isolating clones containing DNA flanking the transposon. The system also promotes the rapid isolation of large DNA fragments, allowing the isolation of clusters of virulence genes.

The broad host range plasmid-transposon construct pTn*Mod*-OGm consists of a cognate transposase outside of the transposon's inverted repeats that permits the minitransposon to integrate into the target DNA without its transposase and prevents further DNA rearrangements (3). This system is constructed with a pUC (pMB1/ColE1) conditional origin of replication within the transposon, which limits its replication to *Escherichia coli* and some closely related species (3). In addition, rare restriction endonuclease sites are incorporated near the inverted repeats, which are features that promote the rapid cloning of DNA adjacent to the transposon's site of insertion. Oligonucleotide primers were designed based on the nucleotide sequence of the alkaline phosphatase gene without the signal sequence ('*phoA*) of *E. coli* and used to amplify a 1.7-kb product from pRT733 (Tn*phoA*) (4). The sequence of the primers used are as follows (*Kpn*I restriction sites are underlined): Pho-5′, 5′-GCGGTACCCTGACTCTTATACACAAGTAGCGT-3′ and Pho-3′, 5′-GCGGTACCCAGGCAATCACTCATGTAGGT-3′. The 1.7-kb PCR product was digested with *Kpn*I and ligated to the *Kpn*I site of pTn*Mod*-OGm creating the 6375-bp construct pmini-O*phoA*

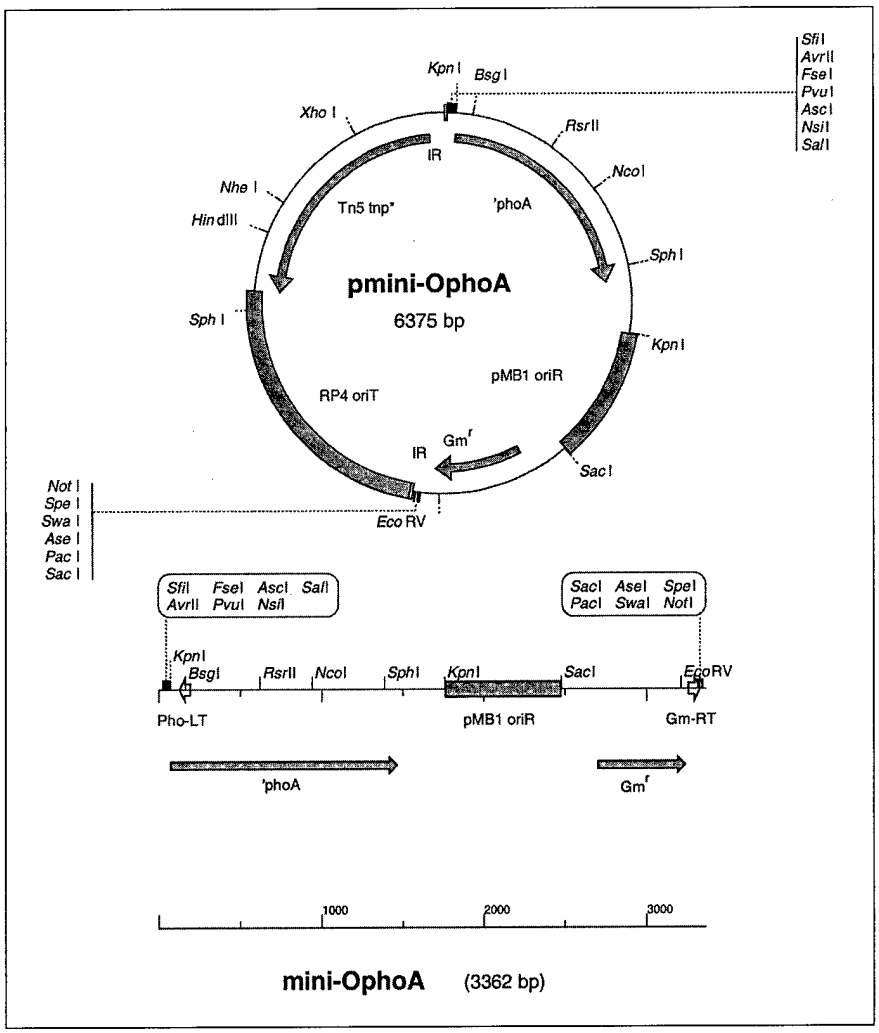


Figure 1. Schematic representation of the pmini-O*phoA* construct and the mini-transposon, mini-O*phoA*, following transposition into the host chromosome. '*phoA*, *E. coli* alkaline phosphatase without the signal sequence and expression signals; pMB1 oriR, narrow host range origin of replication; Gm^r, gentamicin resistance cassette; IR, Tn5 inverted repeats; RP4 oriT, origin of transfer; Tn5 tnp*, Tn5 transposase; Pho-LT and Gm-RT, mini-O*phoA* universal sequencing primers (open arrows).

(Figure 1). Orientation of the *'phoA* gene was confirmed following digestion of the construct with *Sph*I. Insertion of *'phoA* does not disrupt its modular arrangement; the gentamicin resistance cassette can be released with *Sac*I and replaced with alternative antibiotic cassettes, allowing the use of this system in bacteria that are naturally resistant to a variety of antibiotics (3).

The minitransposon derivative mini-O*phoA* (3362 bp) is smaller than Tn*phoA* (7733 bp), and the absence of the cognate transposase confers a higher stability upon the transposon once it has integrated within the target DNA (Figure 1). The presence of the conditional origin of replication and multiple cloning sites within the inverted repeats promotes the efficient recovery of DNA flanking the transposon compared with other Tn*phoA* systems (2,4). Sites for rare-cutting restriction enzymes within the multiple cloning sites will produce clones that contain large segments of the genome. The close proximity of cloning sites greatly enhances sequencing of DNA flanking the transposon by virtue of the mini-O*phoA* universal primers, Pho-LT and Gm-RT.

The mini-O*phoA* universal primers are designed to nucleotide sequences adjacent to the multiple cloning sites (Figure 1). The sequence of the mini-O*phoA* universal primers are as follows: Pho-LT, 5′-CAGTAATATC-GCCCTGAGCAGC-3′ and Gm-RT, 5′-GCCGCGGCCAATTCGAGCTC-3′. The *phoA*-fusion joint of flanking clones derived from *Ase*I, *Spe*I, *Pac*I, *Swa*I and *Not*I digests can be sequenced using the primer Pho-LT, and upstream DNA sequence can be determined using the Gm-RT primer. The converse can be applied when genomic DNA is digested with *Sfi*I, *Fse*I, *Asc*I, *Sal*I, *Avr*II, *Pvu*I and *Nsi*I when the primer Gm-RT is used to sequence the transposon-fusion joint, and the downstream DNA sequence can be determined using the Pho-LT primer. The application of the mini-O*phoA* universal primers promotes the rapid sequencing of flanking DNA.

The plasmid-transposon construct was transferred into the nalidixic acid- and zeocin-resistant phosphatase-negative derivative of *Burkholderia mallei* strain ATCC 23344 by conjugation with *E. coli* SM10 (pmini-O*phoA*) and selected on agar incorporated with gentamicin (5 µg/mL), BCIP (80 µg/mL), zeocin (5 µg/mL) and nalidixic acid (75 µg/mL). Approximately 1000–2000 transconjugates were isolated per mutagenesis, with a frequency of phosphatase expressing (PhoA+) mutants of 2%. Chromosomal DNA was isolated from PhoA+ mutants by using the minichromosomal preparation protocol described by Ausubel et al. (1). One microgram of total genomic DNA was

digested with either *Not*I, *Sal*I, *Avr*II, *Spe*I or *Pvu*I. Following digestion, the enzyme was heat inactivated, and the DNA was ethanol precipitated and resupended in 20 µL sterile distilled water. Ten microliters of this suspension were self-ligated in a total volume of 20 µL overnight at 16°C. Four microliters of the resulting ligation mixture were subsequently transformed into 40 µL competent *E. coli* DH5α™ (Life Technologies, Rockville, MD, USA).

Isolation of both transconjugates and flanking clones was approximately tenfold more efficient using pmini-O*phoA* than using the pRT733 (Tn*phoA*) system. Flanking clones were sequenced using the mini-O*phoA* universal primers, Pho-LT and Gm-RT, which enabled sequencing of the *phoA*-fusion joint and DNA up or downstream of the transposon's site of insertion. Blastx searches of the PhoA-fusion proteins showed homology to secreted bacterial proteins and demonstrated that this system successfully isolates defined mutations in secreted proteins. The mini-O*phoA* system has been used successfully to isolate defined mutations in secreted proteins of *B. pseudomallei* and *B. thailandensis* (M.N. Burtnick, A. Bolton, P. Brett, D. Watanabe and D.E. Woods, unpublished results).

Isolation of flanking clones that contain large segments of DNA can allow for the rapid identification of virulence loci because virulence-associated genes are often clustered. Moreover, sequencing of adjacent genes can often ascribe a putative function to the *phoA*-fusion protein and assign a foundation for virulence studies when *phoA*-fusion proteins exhibit no or low homology to other known proteins. An additional use of the pTn*Mod*-OGm has been suggested by Dennis and Zylstra (3), who propose that plasmid-transposon construct-generated libraries are a relatively swift and simple alternative to the construction of cosmid libraries. This notion is based on the use of the rare-cutting restriction enzymes that will produce clones that contain large segments of the genome. This approach involves the isolation of genomic DNA from pools of Tn*Mod*-OGm transconjugates, cleavage with a rare-cutting restriction enzyme, self-ligation and transformation into a permissive *E. coli* host. A similar approach could conceivably use mini-O*phoA* and confer the selection of libraries that encode secreted proteins.

In conclusion, we have constructed a broad host range self-cloning minitransposon *phoA* gene-fusion system that promotes the rapid identification of defined mutations in secreted proteins of Gram-negative bacteria. The mini-O*phoA* is a versatile tool that enhances the isolation of virulence genes and promotes the recovery of large segments of DNA flanking the transposon, thus allowing the identification of clusters of virulence genes and providing an alternative approach for the production of cosmid libraries.

REFERENCES

1. **Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (Eds.).** 1987. Current Protocols in Molecular Biology. Greene Publishing and Wiley-Interscience, New York.
2. **de Lorenzo, V., M. Herrero, U. Jakubzik and K.N. Timmis.** 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. *172*:6568-6572.
3. **Dennis, J.J. and G.J. Zylstra.** 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram negative bacterial genomes. Appl. Environ. Microbiol. *64*:2710-2715.
4. **Taylor, R.K., C. Manoil and J.J. Mekalanos.** 1989. Broad-host-range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. *171*:1870-1878.

*This work was supported by Department of Defense contract no. DAMD 17-98-C-8003 and the Medical Research Council of Canada. We are grateful to Dr. J. Dennis for providing us with pTn*Mod-*OGm.* *Address correspondence to Dr. Donald E. Woods, 3330 Hospital Drive, Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, Calgary, AB T2N 4N1, Canada. e-mail: woods @ucalgary.ca*

Received 21 March 2000; accepted 25 May 2000.

Alexandra J. Bolton and Donald E. Woods
University of Calgary Health Sciences Centre
Calgary, AB, Canada

Personnel Supported by this Research

Dr. Alexandra Bolton
Dr. Chanwit Tribuddharat
Dr. Richard Moore